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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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EFFECT OF CERTAIN TISSUE EXTRACTS ON RED BLOOD CELL REGENERATION.*

By ANDREW JENEY and JAMES W. JOBLING.

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(Received for publication, August 16, 1927.)

While most of the anemias in human beings are due to some well known cause, and are for this reason looked upon as secondary, there are instances in which the cause cannot be determined, though the blood changes indicate that even these are of the secondary type. For this reason most of the experimental work done in the past in the attempt to obtain more information as to the cause of anemia in human beings, particularly pernicious anemia, has been based on the supposition that it is due to some form of intoxication. Recently, however, evidence has been furnished indicating that the course of experimental anemias in animals, as well as of pernicious anemia in human beings, may be favorably influenced by special diets. This suggests that some of the obscure anemias may be due to the lack of some substance in the body which is essential to normal blood formation and which may be furnished in the food. The recent work showing that calcium metabolism may be affected by various means suggests that the same may be true of iron.

Experiments planned to show the effect diet may have in producing anemia in animals have not been very fruitful. Many such attempts have been made with diets poor in iron, or in some other supposed essential, but the results were usually negative. Malnutrition in animals can be easily produced, but this is never accompanied with the characteristic blood changes seen in pernicious anemia.

Usually it is thought that the oxygen requirement of the body is the most important controlling influence in determining the number of red blood cells in the circulating blood, and this belief has received

* This work was aided by a grant from the Eli Lilly Company.

considerable support from the experiments made on animals and human beings which had been kept at high altitudes. However, neither this work nor the work with diet proves that there may not be some other regulatory mechanism, possibly located in the liver.

In 1904 Perrin (1) reported good results in the treatment of three cases of severe anemia, which were associated with hepatic cirrhosis, by adding fresh liver to the diet. The results obtained with the feeding of liver by Whipple (2) and his associates in the treatment of experimental anemia in dogs and by Minot and Murphy (3) in the treatment of pernicious anemia, have made it evident that this organ plays an important part in red blood cell formation. Recently Whipple and Robscheit-Robbins (4) have found that water and alcoholic extracts of liver fed to dogs with long continued anemia likewise promote red cell regeneration. It will be noted that all of Whipple's experiments were made with dogs which had a long continued anemia.

The belief has been prevalent for a number of years that the spleen in the adult, through a stimulating action on the bone marrow, influences blood formation. Though this view is not accepted by all observers, it is well known that it is directly concerned in blood formation during fetal life, and particularly during adult life, in the processes of red blood cell destruction. But what is the evidence in support of the belief that the spleen produces an internal secretion which regulates the number of red blood cells in the circulation? For a detailed discussion of the functions of the spleen the reader is referred to the recent papers by Eddy (5) and Krumbhaar (6).

Brinckmann (7) found that feeding normal rabbits with fresh ox spleen caused a reduction in the red and white blood cells. Zalinski (8) and Danilewski (9) reported a marked increase in the number of red cells by a single intraperitoneal injection of splenic extract, but Patou, Gulland and Fowler (10) were unable to confirm these observations. Krumbhaar and Musser (11) also report an increase in the red count following the injection of a fresh splenic extract. This increase lasted 1 or 2 days. Eddy (5) in his experiment used saline extracts of powdered dried spleen and protein-free extracts of fresh and dried spleen. The red cell counts were made at half hour intervals and continued 4 to 5 hours after the injection. Each of his preparations caused a decrease in the number of red cells in the circulation. On the other hand, Leake and Bacon (12) report that the feeding of fresh spleen, or the intravenous injection into rabbits of saline solution extracts of fresh spleen, caused a fall in the number of circulating erythrocytes within 24 hours. It went to 7 per cent above normal after the 3rd day, but soon fell to normal when administration was discontinued.

Methods.

Extracts of liver, spleen, gastric mucosæ and hemoglobin solution were used in the experiments. The gastric mucosæ were obtained from pig stomachs, and the

other organs from rabbits or calves. For preparing the protein-free extracts the tissues were ground to a pulp by passing repeatedly through a meat grinder. They were then extracted with an equal volume of 95 per cent alcohol to which had been added 0.5 per cent acetic acid. After standing overnight in the ice box the mixture was passed first through gauze and then through filter paper until the filtrates became clear. The tissue remaining was extracted twice in the same manner with 75 per cent alcohol. The filtrates from the three extractions were combined and evaporated to dryness at a temperature not exceeding 45°C., under negative pressure. The partially dried mass was then extracted with 75 per cent alcohol, filtered and the filtrate again evaporated to dryness. For one experiment, Dr. Zucker, of this department, kindly prepared for us a fat-soluble non-saponifiable extract of the liver.

In addition to the above, simple saline extracts of the tissues were used. These extracts were prepared by grinding up the fresh organ with saline solution in a mortar. Ten parts of saline solution were used to 1 of the tissue. The mixture was kept in the ice box overnight and then cleared either by filtration through sterile filter paper or by centrifugalization. The hemoglobin solution was made by laking 15 parts of washed rabbit red cells with 25 parts distilled water and cleared by centrifugalization.

Rabbits were used in all experiments. They were made anemic by bleeding from the heart; ether anesthesia was used. If necessary to save the life of the animal, the blood lost was replaced with saline solution. The blood removed constituted about 3 per cent of the body weight, or about 60 per cent of the total volume. Several blood counts, hemoglobin determinations and reticulated cell counts, were made before the bleeding and at frequent intervals afterwards. At the end of each experiment autopsies were done, particular attention being paid to the organs concerned in blood destruction and regeneration. The details of the autopsy findings will be reported subsequently.

The normal red count in our rabbits was found to be between 5 millions and 6 millions, which the bleeding usually lowered to about 2.5 to 3 millions. Only those animals with low red cell counts were used in the experiments. The animals were all kept under the same conditions as to light, food and temperature, and were weighed regularly. Our interpretation of the results of the injection of the different substances was based upon the rapidity with which the normal count was restored, as compared to a series of similar untreated animals. Each of the experiments has been repeated several times during the last few years.

In our preliminary experiments we found that the red count in the control animals losing about 60 per cent of their total blood was usually

not restored to normal until about 3 to 4 weeks subsequent to the operation, though evidence of regeneration became quite evident at the end of the 2nd week. There was also a marked loss in weight during the first 2 weeks. With the increasing red count there was an increase in reticulocytes.

The first experiment was planned to show the effect of protein-free extracts of the liver, spleen and stomach on normal animals. Two rabbits were used for each extract. The freshly prepared extract in doses of 5 mg. per kilo of body weight was given subcutaneously on 6 successive days in each week. As no effect was apparent after the 1st week, the dose was then increased to 10 mg. and this continued for a period of 25 days.

The animals after receiving the liver extract showed some increase in red cells after 30 days, but the increase was so slight that it was within the limits of normal variations in the red cell count. There was an increase in the neutrophilic leucocytes. Those receiving the stomach extract showed a very slight decrease. On the other hand, the rabbits receiving the spleen extracts showed a decrease of 1.8 and 3 millions, respectively. The number of reticulocytes was increased in the animals receiving the liver extracts, but was not affected by extracts of spleen and stomach. There was some increase in the lymphocytes.

This experiment was repeated several times with the same results as regards the liver and stomach extracts. In subsequent experiments, however, the spleen extract, whether protein-free or from freshly prepared cell emulsions in salt solution, was more active in causing a decrease in the red cell count. In the animals receiving the spleen extracts the per cent of reticulocytes, regardless of the degree of anemia, was always low, sometimes considerably below normal. There were no changes in the white blood cells which could be ascribed to the extracts.

The preceding experiment was repeated with animals which had been made anemic by one large bleeding. 2 days subsequent to the bleedings the red cell counts in this series of animals were between 2.5 and 3.2 millions. Three were left as controls, while the others received 10 mg. doses of the extracts of liver, spleen and stomach. All injections were made into the subcutaneous tissues.

Blood regeneration in the animals receiving the liver extract appeared to be a little more active than in the controls, and the reticulocytes increased earlier and were somewhat more numerous. The neutrophil leucocytes also were somewhat increased. The extracts of the gastric mucosæ appeared to exert no influence on regeneration. In the animals receiving the spleen extract there was very little red cell formation. When small doses were given there was a prolonged period during which there was little evidence of regeneration; but with larger doses the red count was still further depressed. The number of reticulocytes in these animals was very low.

A new series of rabbits was made anemic by bleeding from the heart and then injected with extracts, both saline and protein-free, prepared from the liver and spleen of rabbits which had been made anemic by repeated bleedings. The animals from the organs of which the extracts were prepared were bled every other day for 18 days. During this period a little more than 200 cc. of blood was taken from each animal, so that the final red cell count in each was a little less than 2 millions, and the hemoglobin approximately 20 per cent. The average number of reticulocytes for the four animals in the beginning was .5 per cent; after 13 days 7 per cent, and after the final bleeding, approximately 21 per cent. The animals were bled to death under ether anesthesia and the organs used for the preparation of extracts. Protein-free extracts were prepared from the livers of these animals. Because of their small size we were compelled to rely on saline extracts of the spleens. From the blood obtained at the last bleedings, hemoglobin extracts were prepared. The methods used in preparing these extracts have been described.

The hemoglobin solution was injected every other day into the peritoneal cavity of the rabbits in doses of 10 cc. The spleen extract was given in doses of 2 cc. and the liver extract in doses of 10 mg. per kilo, both subcutaneously.

Again, in this experiment, the spleen extract rabbits showed practically no evidences of any ability to generate red cells. The reticulocytes were not increased above the normal, which suggests that the effect noted above may be due to an inhibiting action which the spleen extracts have on blood regeneration, rather than a destructive action, which we at first thought probable.

On the other hand, the rabbits receiving the hemoglobin and liver extracts did show active red cell regeneration by the end of the 2nd week, and the per cent of reticulocytes was somewhat higher than that seen in the controls for the corresponding period.

In the next experiment we tested the action of a fat-soluble non-

saponifiable liver extract kindly prepared for us by Dr. Zucker of this department. In addition, we again studied the action of the protein-free extracts of the liver and spleen. This spleen extract had been kept in the ice box for more than 5 months.

The results of this experiment indicated that the fat-soluble non-saponifiable liver fraction was inactive, while the spleen extract, in spite of its age, showed the usual inhibiting effect. The blood regeneration in the rabbits receiving the non-protein liver extract was quite active.

Though none of the extracts used appeared to be very active in stimulating blood regeneration, yet there was some indication that the normal red cell count was restored a little more quickly in the animals which had received the liver extract and hemoglobin solution than in the untreated anemic controls. As extracts prepared from the organs of animals made anemic by bleeding were inactive, our next experiment was made with extracts prepared from the organs of animals made anemic by destroying the red cells within the body. This was accomplished by giving intravenous injections of distilled water. The injections were given five times in 2 weeks. One animal received 140 the other 205 cc. of water. The purpose of the experiment was to see if the organs of animals made anemic in this manner would contain the supposedly stimulating end-products of the red cells. Briefly, the results of the injection of these extracts showed no difference from the preceding.

While preparing these animals we were surprised to note the amount of distilled water which could be given in this manner without causing death. In subsequent experiments it was found that rabbits would frequently survive doses of 80 cc. and 120 cc.

DISCUSSION.

In part of our experiments with liver extracts we obtained results which indicated that they contained active substances, but while red cell regeneration began early in all the liver extract animals, a few of the controls showed the same early regeneration. In addition, the differences were not striking in any instance and were within the limits of experimental error. For this reason they were repeated several times over a period of more than 3 years, with the results mentioned.

Of the different extracts used, those prepared from the livers of anemic animals acted best, as the red count in the rabbits receiving these preparations apparently was restored to normal more rapidly than in the controls, and the reticulocyte count was definitely higher.

On the other hand, the effect of the various spleen extracts was quite striking. Where the amounts given were small, the regeneration of blood was much slower than in the control animals; while with large doses there was a further decline in number of red cells. The reticulocytes were always much fewer in number. It is possible that we

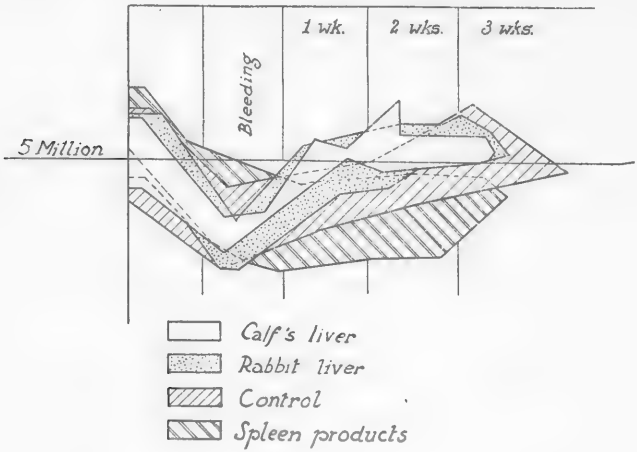

CHART 1.

were dealing with a poison, but the low reticulocyte count suggests inhibition of red cell formation rather than cell destruction.

In the body of the paper we have given no tables showing red cell counts, hemoglobin, etc. Had the results been more positive in character, they would have been included, but under the circumstances it seemed unnecessary to do so. The chart shows more graphically the results we obtained in our experiments with liver and spleen extracts, and represents the averages obtained in numerous experiments. It suggests that blood regeneration in the animals which received both rabbit and calf liver extracts is more active than in the controls, but

the difference is not marked. On the other hand, the spleen obviously has an inhibiting effect.

Our results are particularly interesting in view of those obtained with the feeding of liver by Whipple in the experimental anemias of dogs, and by Minot and Murphy in pernicious anemia. Several explanations of the discrepancies are possible. First, rabbits may not respond to the liver treatment; second, the active substance may have been destroyed by the heat used in drying our alcoholic extracts, though it did not exceed 45°C.; third, our preparations were given by subcutaneous injection, their's by mouth; fourth, our animals were made anemic by a single large bleeding, while Whipple's experiments were conducted with animals having a chronic anemia produced by repeated bleedings.

It seems not improbable that the marrow of animals with a long continued anemia will be hyperplastic and react more readily to the presence of a stimulating substance than those with an acute anemia produced by a single large bleeding. In the former case the marrow is prepared to act, in the latter case it is not.

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STUDIES ON THE MODE OF SPREAD OF B. ENTERITIDIS MOUSE TYPHOID INFECTION.

I. Native Epidemicity.

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GENERAL INTRODUCTION.

Our experimental studies of epidemics have been confined thus far to two native animal diseases: mouse typhoid, caused by an organism of the animal paratyphoid B group, *B. pestis caviæ*,[1] and a respiratory infection of rabbits, *Bact. lepisepticum* snuffles and pneumonia. Mouse typhoid, being readily adapted to laboratory manipulations, has yielded data bearing upon its prevalence and mode of spread; rabbit snuffles, notwithstanding the technical difficulties encountered, has been of use in confirming the data and extending their application (1, *a*).

The scope of experimental epidemiology may be enlarged by extending the investigations to still other animal diseases. With this end in view, we have turned our attention to a third native infection, a variety of mouse typhoid induced by a bacillus of the enteritidis group. This infection is wide-spread among rodents (2), and occurs also in man as a form of food poisoning (3). Mice affected show gross and microscopic lesions resembling those occurring in the paratyphoid B infection (4). Indeed, the major features of these two diseases in mice are similar.

However, two rather special characteristics of the enteritidis infection in mice make a study of the disease of special interest from the point of view of experimental epidemiology. The first of these is the fact that a high titre bacteriophage may be obtained readily from a large per cent of infected animals; the second is that under relatively

[1] This organism is also designated *B. aertrycke* and *B. paratyphi.*

simple conditions, the typical smooth colony forms of *B. enteritidis* change to mucoid and rough colony variants. Similar bacteriophage and transformation processes have been considered, of late, to play such an important part in determining the prevalence of other diseases, that it seemed desirable to study the phenomena as they occur in *B. enteritidis* mouse typhoid infection and determine, if possible, the extent to which they influence its spread.

The studies to be described are divided as follows: (1) Bacteriological tests on mice from various scattered populations to determine the prevailing colony types of paratyphoid-enteritidis bacilli. The results of these tests are summarized in this paper. (2) Attempts were made to determine the conditions under which "spontaneous" transformations of colony types occurred. These experiments are described in the next paper. (3) In Papers III and IV of the series, comparative studies of bacilli from the various colony types were carried out with special attention to differences in virulence. From these results, a working hypothesis was formulated to explain the mechanism of colony transformation. (4) Finally, an elaborate study of several populations involving several thousand mice spontaneously infected with *B. enteritidis* and a Friedländer-like organism has been carried on over a period of 2 years, to determine at various interepidemic, preepidemic, and epidemic periods, the colony types and relative virulence of the specific bacteria, and the effect of changes in carrier rate, season, and experimental alteration in population resistance on the spread of disease. These results are to be published later.

Types of B. enteritidis Recovered from "Spontaneously" Infected Mice.

Apparently there is general agreement among recent investigators that in native paratyphoid-enteritidis infection, the smooth colony type of organism predominates at all times.

Thus Topley encountered only smooth types of *B. gaertner* and *B. aertrycke* during his observations of experimental mouse typhoid epidemics. Thomas describes paratyphoid-enteritidis bacilli isolated from guinea pigs as forming, in general, the smooth type of colony, although one strain of *B. enteritidis* appeared rough (2, *c*). Savage and White, in an extensive investigation of the Salmonella group, state that "in direct platings from pathological material, colonies of

Salmonella bacilli are, as a rule, wholly smooth. On occasion, however, roughness may be detected in these first cultures" (3, *d*). Nelson, describing a typhoid epidemic among guinea pigs, states that smooth colony types predominated; occasionally, a few mucoid variants were encountered.

During the past 5 years, one of us (Pritchett) has tested a number of batches of mice obtained from various sources to detect the presence of mouse typhoid bacilli. The results of these tests, which include the species and colony type of bacillus obtained, are summarized briefly in Table I and may be stated as follows:

Group 1. Hagedoorn Albinos.—10 mice received from Professor Hagedoorn of California in June, 1922. 1 was dead on arrival and at autopsy *B. enteritidis* was recovered from the blood and feces. A year later a mouse of this strain, a descendant of the original 10, died and was found at autopsy to harbor the smooth colony type of *B. enteritidis*.

Group 2. Baltimore Albinos.—100 mice received from a dealer in Baltimore. These mice had been strain inbred for a year and a half. 1 mouse was dead on arrival and other mice died at intervals of a few days. The smooth type *B. enteritidis* was cultured from the blood and feces of the 10 or 12 autopsied.

Group 3. Annandale Hybrids.—Composed of black, brown, yellow, and white mice. 51 of these mice were received from Annandale, New York, in November, 1922. 3 were dead on arrival, and 1 other died the next day; all were found to harbor *B. pestis caviæ* in the blood and feces. Of the 47 which lived, 12 were found to be fecal carriers of *B. pestis caviæ* so that of the entire group about 23 per cent were infected with this organism; smooth colony types alone were recovered.

Group 4. Bagg Albinos.—Of 45 mice sent from Cold Spring Harbor, New York, in December, 1922, 1 was dead on arrival and was found to harbor *B. pestis caviæ* in the blood, feces, and internal organs. Of the 44 which lived, 36 were fecal carriers of *B. pestis caviæ*, so that 82 per cent of the original lot of 45 were shown to be infected with this organism; only smooth types were obtained.

Group 5. Lathrop Blacks.—45 mice sent from Cold Spring Harbor, New York, in December, 1922. Two separate fecal cultures of all the mice failed to show any mouse typhoid bacilli.

Group 6. Lathrop Browns.—100 mice sent from Cold Spring Harbor, New York, in the early spring of 1923. 1 mouse was dead on arrival and 10 or 12 succumbed during the 1st week; all showed the presence of smooth colonied *B. pestis caviæ* in the blood and feces.

Group 7. Louvain Albinos.—7 mice were received from the University of Louvain, Belgium, in May, 1924, of a stock bred in that laboratory for 3 or 4 years. By the end of May, all but 1 had died, and the single survivor died later. From three autopsies heavy growths of smooth type *B. enteritidis* were obtained from the spleen and feces.

TABLE I.

Prevalence of Mouse Typhoid Bacilli in Different Mouse Populations.

Group	Description of mice examined	Source of mice and date of examination	No. in lot	Per cent negative for mouse typhoid	Mouse Typoid I (*B. enteritidis*)			Mouse Typhoid II (*B. pestis caviæ*)		
					No. of positive	No. of smooth colonies	No. of mucoid colonies	No. of positive	No. of smooth colonies	No. of mucoid colonies
1	Hagedoorn albinos	California, 1922	10	?	1 mouse	1 mouse	0	0	0	0
2	Albinos	Baltimore, 1922	100	?	10 mice	10 mice	0	0	0	0
3	Hybrids, black, brown, yellow, and white	Annandale, N. Y., 1922	51	77	0	0	0	23 per cent	All	0
4	Bagg strain albinos	Cold Spring Harbor, N. Y., 1922	45	18	0	0	0	82 per cent	All	0
5	Lathrop strain black	Cold Spring Harbor, N. Y., 1922	55	100	0	0	0	0	0	0
6	Lathrop strain brown	Cold Spring Harbor, N. Y., 1923	100	?	0	0	0	10 mice	10 mice	0
7	"Louvain" albinos	Louvain, Belgium, 1924	7	?	3 mice	3 mice	0	0	0	0
8	Rockefeller Institute farm albinos	Princeton, N. J., 1923	100	?	10 mice	10 mice	0	0	0	0
9	Rockefeller Institute "Lynch" black agoutis	New York, N. Y., 1922	50	76	22 per cent	All	0	2 per cent	0	0
10	Albinos	Pennsylvania, 1925	64	88	22 per cent	100 per cent	0	0	0	0
11	Rockefeller Institute breeding room albinos	1922–1926	Many thousands	?	Less than 0.01 per cent	All	0	0	0	0

12	Pritchett experimental epidemic	1925	80	60	Spontaneous 37.5 per cent infection	96.4 per cent	14.2 per cent	2.5 per cent experimental infection		
13	Webster experimental epidemics	1925–1927	4000+	Spontaneous M. T. I infection		99+ per cent	1 per cent or less	0	0	0

Group 8. Princeton Albinos.—100 mice received from the Department of Animal Pathology in October, 1923. 1 mouse was dead on arrival, but autopsy cultures showed no mouse typhoid bacilli. Within 2 or 3 days others died and showed the presence of smooth *B. enteritidis* in the blood and feces.

Group 9. Lynch Black Agoutis.—50 mice obtained from The Rockefeller Institute cancer stock in November, 1922. Three fecal cultures were made on the 50 mice between November 8th and December 4th. 11 (22 per cent) were found to be fecal carriers of smooth *B. enteritidis*, while 1 mouse carried smooth type *B. pestis caviæ*.[2]

Group 10. Pennsylvania Albinos.—64 mice obtained from a Pennsylvania breeder in October, 1925. The mice began to die immediately, about 50 per cent succumbing in the first 10 days after arrival. 22 per cent of these mice showed the presence of smooth *B. enteritidis*, either in the feces during life or in the blood and organs at autopsy.

Group 11.—The mouse breeding room at The Rockefeller Institute, New York, supplies 500 to 1000 mice a month. The population of this room is entirely free from *B. pestis caviæ* infection, so called Mouse Typhoid II, but does yield an occasional carrier of the smooth type of *B. enteritidis*, Mouse Typhoid I. Estimates of one 6 month period showed the percentage incidence of carriers to be less than 0.01 (1, *b*).

Group 12.—A group of 80 mice which had survived a series of experimentally induced epidemics of *B. pestis caviæ* was found at autopsy to be infected with both the paratyphoid ($2\frac{1}{2}$ per cent) and the enteritidis organisms ($37\frac{1}{2}$ per cent). All the *B. pestis caviæ* colonies were smooth; while of the *B. enteritidis* infected animals, 14.2 per cent showed an occasional mucoid colony on the culture plates (1, *b*).

Group 13.—Finally, from groups of mice totalling over 4000 in number, among which *B. enteritidis* typhoid epidemics have been occurring, there have been recovered from autopsy cultures, with few exceptions, only smooth colony types (see introductory Paragraph 4, Division 4).

SUMMARY.

Thirteen batches of mice from nine different sources were tested for the presence of mouse typhoid bacilli. Individuals from nine of the groups were found to be infected with the *B. enteritidis* type, four with the paratyphoid B, one with both, and one with neither type. With two exceptions, smooth type colonies alone were found. These results are in conformity with similar observations

[2] The population from which these mice were drawn is entirely separate from the regular Institute breeding room. Lynch has already reported the occurrence of two mouse typhoid epidemics among the cancer stock, that of 1918–19 being due to *B. enteritidis*, and that of 1920–21 to *B. pestis caviæ* (2, *e*).

as reported by others and confirm the general belief that smooth colony types of paratyphoid-enteritidis bacilli prevail throughout the various stages of rodent typhoid infection.

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STUDIES ON THE MODE OF SPREAD OF B. ENTERITIDIS MOUSE TYPHOID INFECTION.

II. Effects of External Conditions on the Occurrence of Smooth, Mucoid, and Rough Colony Types.

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Plate 33.

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The observations on the prevalence of various bacterial types in spontaneous mouse typhoid infection (1, *a*) indicate that the smooth colony forms of paratyphoid-enteritidis bacilli are most common during all the various stages of the disease and the presence of a mucoid or rough colony variant is an exceptional occurrence. Hence it is questionable whether such bacterial transformations, or indeed any bacteriophagic phenomena, influence the extent and severity of the disease. However, this general subject has received so much attention of late in the studies of d'Hérelle (2), Arkwright (3), Hadley (4), and others, that it seemed necessary to study further the problems involved. It is intended in this paper to describe certain conditions under which the transformations of *B. enteritidis* occur from smooth to variant colony forms, and from variant back to smooth type colonies; and later, to offer a partial explanation of the nature of the process.

Cultures were obtained from spontaneously infected mice from various sources (1, *a*). One of these strains (No. 3) has been studied by Amoss and Haselbauer (5), Thomas (6), and Sakai (7), and placed in the enteritidis group.[1] But, chief attention has been given to

[1] This same culture has been used by Theobald Smith and Nelson (*J. Exp. Med.*, 1927, xlv and xlvi) in identifying organisms from an epidemic of guinea pig typhoid. The mouse typhoid (M. T. I) strain used by them for purposes of identification is our Strain 3. They have designated it *B. paratyphi*, whereas we consider it to belong in the *B. enteritidis* group.

strains derived from certain populations of mice at The Rockefeller Institute, among which mouse typhoid has prevailed during the past 2 years in endemic or epidemic form (1, *a*, Group 13). The cultures were quite similar to one another and to those from outside sources (1, *a*, Table I). In brief, they consisted of Gram-negative, motile rods, growing diffusely in broth, fermenting dextrose, maltose, xylose, rhamnose, and mannite, but not lactose, saccharose, salicin, and inosite. No indole was produced; lead acetate was blackened. Stock *B. enteritidis* sera agglutinated the strains to titre and sera prepared from several of our own strains brought about maximum agglutination in all.

Colony Formation.

Direct Plating from Autopsy Material or from Fecal Carriers.—As stated previously (1, *a*, Group 13, Table I), several thousand mice have been examined bacteriologically during the course of our observations on special mouse populations, and found to harbor *B. enteritidis*. The cultures, when taken directly from the animal, streaked over plain agar plates, and incubated at 37°, formed typical smooth colonies. Occasionally, however, if the plates were left at room temperature—23°C.—for 12 hours or more at any time during the period of observation, mucoid colonies developed, or the smooth forms became surrounded by a ring of mucoid growth. Still more rarely and under different circumstances, an occasional rough colony was encountered.

Serial Passage of Smooth Colony Types on Agar and in Broth.—The natural tendency for *B. enteritidis* to form smooth type colonies can be interfered with in many ways.[2] But in order to analyze the transformation process as it occurs "spontaneously," we decided to observe the relatively simple conditions which might be encountered normally in nature and in the laboratory.

Experiment 1 shows the effect of direct agar passage at 37° on the type of colony formation.

Experiment 1.—Four mouse strains of *B. enteritidis* were taken directly from the stock meat infusion agar slant and passed serially on meat infusion agar

[2] In Hadley's monograph, the literature on this subject is reviewed at length (4).

plates, pH 7.4. Strain 1 is a single cell culture[3] obtained on December 2, 1925, from a mouse from one of the special populations mentioned above (1, *a*, Group 13). Strain 2 is a similar culture from another mouse dying on the same date. Strain 3 is a single cell culture obtained from a wild mouse found dead in a Rockefeller Institute laboratory in 1921. This strain has been studied by other investigators (5–7). Strain 4 is a single cell culture obtained in August, 1925, from a mouse from one of the special populations (1, *a*, Group 13).

A small amount of the stock culture was taken from each slant and spread over plates containing plain beef infusion agar. After 24 to 48 hours incubation at 37°, the resulting colonies were examined, and one of them picked and spread over another agar plate. This method of passage was carried on eight times for each strain.

TABLE I.

Passage of Smooth Type Colonies of B. enteritidis on Plain Agar Plates at 37° and 23°C.

Date	Passage	Temperature	Strain 1		Strain 2		Strain 3		Strain 4	
			Colony type seeded	Colony type resulting	Colony type seeded	Colony type resulting	Colony type seeded	Colony type resulting	Colony type seeded	Colony type resulting
1926		°C.								
11/15	1	37	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
		24	"	"	"	Mucoid	"	"	"	"
11/17	2	37	"	"	"	Smooth	"	"	"	"
		24	"	"	"	Mucoid	"	"	"	"
11/19	3	37	"	"	"	Smooth	"	"	"	"
		24	"	"	"	Mucoid	"	"	"	"
11/21	4	37	"	"	"	Smooth	"	"	"	"
		24	"	"	"	Mucoid	"	"	"	"

Eight agar passages of these four single cell, smooth colony strains had no modifying effect on colony type. Plates from each passage showed 100 per cent typical smooth colony forms. Other mouse strains of *B. enteritidis* behaved in a similar manner for as many as twenty passages.

The effect of temperature on colony type formation was then tested (Experiment 2).

[3] Single cell strains were obtained by the use of a technique described by Reimann (8).

Experiment 2.—The cultures used in Experiment 1 were passed in the same manner as before, except that each transfer was made in duplicate. One set of plates was incubated at 37°C.; the other left at room temperature, 23°. A single, well isolated colony from the 37° plate was used for each transfer. The results are shown in Table I.

Strains 1, 3, and 4 remained 100 per cent smooth at both 37° and 23° for four passages. "Smooth" colonies of Strain 2, however, when incubated at 37°, grew out 100 per cent smooth, but when allowed to grow at room temperature at 23°, appeared entirely mucoid. These colonies remained mucoid when transferred at 23°, but invariably reverted to smooth forms when grown at 37°. In all, twenty-one cultures of *B. enteritidis* were tested in this way. Fourteen came from the populations described above (1, *a*, Group 13), four from outside mice, and two from spontaneous guinea pig infections. Three of these twenty-one strains showed this characteristic of smooth growth at 37° and mucoid growth at 23°.

D'Hérelle (2) and Hadley (4) mention a number of investigators who have studied the effect of temperature on bacterial transformation, and more recently, Knorr (9) states that certain paratyphoid strains grown in broth at 22° and transferred to enriched solid media of a certain thickness and pH of 8.0 result in the appearance of mucoid colonies after 24 to 48 hours. However, we have seen no definite experimental evidence relating mucoid colony formation directly to temperature conditions.

From these experiments it is concluded that mouse strains of smooth type *B. enteritidis* tend to remain type-pure if passed from agar to agar at 37°, but that an occasional smooth strain becomes mucoid if grown on agar at 23°. The resulting mucoid colonies will remain so indefinitely if transferred and grown at 23°, but when the temperature is raised to 37°, revert immediately to the smooth form.

True rough colonies were not found on any plates during the serial agar transfer of twenty-one smooth colony strains. Fresh stock cultures were passed, together with others, 2, 4, 8, and 12 weeks, and 2, 4, and 6 months old. Colonies 72 hours and 3 weeks old were also used. Dry plates and plates with considerable surface moisture were tested. Frequently, the dry plates produced atypical rough

colonies after 24 hours incubation, but these became relatively smooth at 48 hours, and when transferred, grew diffusely in broth and appeared entirely smooth on fresh agar plates.

From old broth cultures, however, true rough colony types were obtained.

Experiment 3.—Twenty smooth type mouse typhoid strains of *B. enteritidis* were studied. Strains 1, 2, 3, and 4 have been described in the first experiment. Nos. 5, 6, 7, and 13 to 20 are single cell cultures, obtained from the special populations from July, 1925, to April, 1926. Nos. 8, 9, 10, and 11 came from outside mice dying from "spontaneous" mouse typhoid (1, *a*). These are single colony cultures obtained after repeated isolation. Strains 20 and 21 came from cases of spontaneous guinea pig typhoid.

TABLE II.

Transfer of Smooth Colony Strains from Broth to Agar.

Date	Age	Strain No.																			
		1	2	3	4	5	6	7	8	9	10	11	13	14	15	16	17	18	19	20	21
	days																				
3/22	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3/26	12	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3/28	14	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4/11	28	S	S	S	R	R	S	R	S	S	S	S	R	R	R	S	S	R	R	S	S
4/16	33	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	R	R	R	R	R

S = smooth colony. R = rough colony.

Each culture was transferred from the stock agar slant to 5 cc. centrifuge tubes of beef infusion broth, pH 7.4. After 17 hours incubation at 37°, they were placed at room temperature. 8 days later a drop of sediment from each tube was streaked over the surface of 15 cm. Petri dishes containing plain meat infusion agar. The plates were incubated for 48 hours, and then left at room temperature for 1 week. Similar platings were made on the 12th, 14th, 28th, and 33rd days. All suspected rough colonies were grown in broth to detect the characteristic granular type of growth, transferred to plain agar, grown in sugars, and identified by means of specific sera of a titre of 1:10,000. The results are shown in Table II.

No rough colonies were seen on the 8th, 12th, or 14th day. At 28 days, however, and again at 33, several strains, when transferred to agar, showed numerous typical rough colonies agglutinating in

B. enteritidis serum. Transfers from a single colony to broth grew granularly and three serial passages of single colonies from agar to agar resulted in pure rough type cultures.

When single colonies of the rough variant strains are passed from agar to agar, they remain pure for several transfers, but eventually revert to smooth. When passed from broth to broth, reversion to the smooth type occurs after the first or second passage.

Mucoid and rough colony variants also occur when broth cultures of the smooth colony forms are transferred daily.

TABLE III.

Passage of Smooth Type Colonies of B. enteritidis in Plain Broth at 37°C.

Strains	Colony type seeded	Colony types resulting	Passage								
			1 12/21 1925	2 12/22	3 12/23	4 12/24	5 12/26	6 12/27	7 12/29	8 12/30	9 1/7 1926
No. 1	Smooth	Smooth	++++	++++	+++	+++	+++	+++	++	++	+++
		Mucoid	0	0	+	+	+	+	++	++	+
		Rough	0	0	0	0	0	0	0	0	0
No. 3	"	Smooth	++++	++++	++++	++++	+++	+++	+++	+++	+++
		Mucoid	0	0	0	0	+	+	+	1	1
		Rough	0	0	0	0	0	2 colonies	0	1	1

++++ = 100 per cent; +++ = 75 per cent; ++ = 50 per cent; + = 25 per cent; 1 = 10 per cent.

Experiment 4.—Single cell Cultures 1 and 3 were employed. Both were seeded from the stock strain to beef infusion broth at pH 7.4, and incubated at 37°. After 24 hours, one loop of culture was streaked over the surface of an agar plate and a second loop transferred to a fresh broth tube. 24 hours later the culture in the latter tube was plated and transferred again. In all, nine broth passages and nine sets of agar plate transfers were made. Table III shows the results.

Strain 1 showed only smooth type colonies on agar transfer plates until the third broth passage. Mucoid colonies then appeared in gradually increasing numbers, due probably to the fact that after 24 hours incubation, the plates were left at room temperature. After four passages of Strain 3, mucoid forms appeared, later accompanied by occasional rough colonies.

To summarize briefly it may be stated that (1) most smooth type cultures of mouse typhoid *B. enteritidis* remain smooth when passed on agar; (2) that an occasional smooth strain becomes entirely mucoid when grown on agar at 23° and changes again to smooth when the temperature is raised to 37°, and finally (3) that when smooth types are grown in broth for 3 weeks or more, or transferred daily from broth to broth and plated on agar, rough colonies appear which remain rough for several agar passages, but revert to smooth forms when grown in fresh broth. Apparently, therefore, *temperature* is one factor involved in this kind of reversible transformation from smooth to mucoid colony, and *a fluid state of the media* a factor influencing the reversible transformation from smooth to rough colony form.

Effect of Bacteriophage on Colony Formation.—Many investigators have noted that bacteria grown in the presence of an active lytic agent give rise to secondary cultures which form mucoid and rough colony variants. Indeed, there seems to be general agreement with Arkwright's statement that bacteriophage stimulates normal microbic mutation (3). The results of our own observations, however, while agreeing with the general findings of previous investigators, suggest a different explanation of the process.

Active bacteriophage was obtained by various procedures,—namely, broth passage and filtration of a smooth, "eaten" colony of *B. enteritidis*, taken directly from an autopsy plate, and passage and filtration of smooth type cultures plus mouse intestinal material. The titre of this bacteriophage was found to be 10^{-9} for all of our single cell, smooth colony types of *B. enteritidis*.

Experiment 5 was planned to show the effect of bacteriophage-containing agar on the colony formation of smooth type strains of *B. enteritidis*.

Experiment 5.—300 cc. of agar in flasks were melted, cooled to 48°, and poured into 10 cm. Petri dishes. The agar used for the control plates received 30 cc. of plain broth, pH 7.4, just before pouring; a second flask, 30 cc. of 10 per cent bacteriophage in broth, and a third, 30 cc. of undiluted bacteriophage in broth (titre 10^{-9}).

A small portion of each bacterial culture was taken directly from the stock slant and spread over the surface of two plates each, of plain agar, 1 per cent and

TABLE IV.

Passage of Smooth Type Colonies of B. enteritidis to Plain and Phage Agar at 37° and 23°C.

Date	Temperature	Culture used for inoculation			Colony types appearing on plates		
		Strain	Colony type	Source	Plain agar	Agar + phage 1 per cent	Agar + phage 10 per cent
1926	°C.						
11/23	37	1	Smooth	Stock slant	SS ++++	SR ++ E ++	SR ++ E +
		2	"	" "	SS ++++	SR +++	SR ++
		3	"	" "	SS ++++	SR +	SR + Ring +
		4	"	" "	SS ++++	E Ring +	Ring 2 colonies
	23	1	"	" "	SS ++++	0	0
		2	"	" "	MS ++++	M R ++	M +
		3	"	" "	SS ++++	E 1 colony Ring +	SR + M +
		4	"	" "	SS ++++	MR 4 colonies	Ring + M +
12/9	37	5	"	" "	SS ++++	SR + E +	SR + E + M +
		6	"	" "	SS ++++	SR + E +	SR + E + M 1 colony
		7	"	" "	SS ++++	SR +	SR +
		8	"	" "	SS ++++	SR +	SR + E + M +
	23	5	"	" "	SS	SR +	SR +
		6	"	" "	SS	SR + MR 1 colony	SR + M 1 colony
		7	"	" "	SS	SR + MR +	SR + M +
1927		8	"	" "	SS	SR + MR +	SR + M +
1/3	37	9	"	" "	SS	SR ++++	SR ++ M 1 colony
		10	"	" "	SS	SR +++	SR ++ E + M 1 colony
		11	"	" "	SS	SR ++++	SR ++
		13	"	" "	SS	SR ++	SR + E + M 1 colony
	23	9	"	" "	SS	0	0
		10	"	" "	SS	0	0
		11	"	" "	SS	0	0
		13	"	" "	SS	0	0

SS = typical smooth colony; SR = small, round, blue colony; E = small, wart-like, "eaten" colony; Ring = smooth colony surrounded by mucoid ring; MR = mucoid colony; MS = mucoid colony.

10 per cent bacteriophage agar. One set was incubated at 37°, the other left at room temperature, 23°. Single cell cultures of Strains 1, 2, 3, 4, 5, 6, and 7, and single colony Strains 8, 9, 10, 11, and 13 were used. These stock strains have been described in the previous experiment.

The results of these tests are given in Table IV. All strains grown on plain agar at 37° gave rise to smooth colonies; at 23° Strain 2 formed mucoid colonies (see Experiment 2). Subcultures from these smooth and mucoid colonies proved susceptible to lysis in broth containing bacteriophage and grew poorly or not at all on 10 per cent bacteriophage agar plates. Hence these and all other typical smooth colonies have been designated smooth-susceptible (SS) (Fig. 1), and similar mucoid variants, mucoid-susceptible (MS). On the 1 per cent bacteriophage agar plates there appeared distinctly less growth and a different type of colony. Instead of the typical smooth-susceptible colonies, there arose small, round, bluish, smooth forms. Some of these were wart-like, with irregular, "eaten" edges and raised centers. Organisms from these colonies resisted lysis; hence the colonies were designated smooth-resistant (SR) (Fig. 2). On plates left at room temperature—23°—mucoid- and smooth-resistant colonies with a mucoid ring occurred, in addition to the smooth-resistant forms. These mucoid colonies likewise proved phage-resistant and hence were designated mucoid-resistant (MR) (Fig. 3). Growth on the 10 per cent phage agar plates was still more scanty. On plates incubated at 37° a few scattered smooth-resistant colonies were seen, with an occasional mucoid or ring form. On plates left at 23° there was less growth. Some remained sterile.

Further experiments with other strains gave similar results. In one series, four single cell strains were passed eight times on plain and bacteriophage agar. Each transfer showed the same types of colony variants as described above. From two guinea pig strains, however, rough colonies occurred regularly on phage agar plates. These resisted lysis and were designated rough-resistant (RR) (Fig. 4). Rough-resistant colonies were also obtained with great regularity by transferring smooth-resistant colonies to plain agar for two or three passages. In these two instances, a fluid medium did not seem to be necessary for the production of rough colonies.

TABLE V.

Lytic Action of Bacteriophage on Smooth Type B. enteritidis.

Broth Cultures.

Incubation	Culture dilution 1:10					Culture dilution 1:100					Culture dilution 1:10,000					Culture dilution 1:100,000				
	Control	Phage 1:10	1:100	1:100,000	1:10,000,000	Control	Phage 1:10	1:100	1:100,000	1:10,000,000	Control	Phage 1:10	1:100	1:100,000	1:10,000,000	Control	Phage 1:10	1:100	1:100,000	1:10,000,000
	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.	G.
hrs.																				
3	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	+	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
5	+1	0	0	0	1	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0
6	++	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0
7	++1	0	0	0	0	+1	0	0	0	+1	0	0	0	0	0	0	0	0	0	0
12	++++	1	1	0	0	+++	0	0	0	0	++1	0	0	1	1	+	0	0	1	1
20	++++	++	++	++	++	++++	+	+1	++	+1	++++	0	0	++R	+	+++	0	0	0	0
24	++++	+++	+++	+++	+++	++++	+	++	+++	+++	++++	0	0	+++R	++	++++	0	0	0	0
27	++++	++++	++++	++++	++++	++++	++R	++++	++++	++++	++++	0	0	++++R	++++	++++	0	0	0	0
30	++++	++++	++++	++++	++++	++++	++++R	++++	++++	++++	++++	0	0	++++R	++++	++++	0	0	1R	1R
48	++++	++++	++++	++++	++++	++++	++++R	++++	++++	++++	++++	0	0	++++R	++++	++++	0	0	++++R	+1
72	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	++++R	++++	++++	0	0	++++	++++

Asparagine—Salt Cultures.

3	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	+	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	+	0	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
6	+	0	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
7	+1	0	0	1	+1	+	0	0	1	1	0	0	0	0	0	0	0	0	0	0
12	++	0	0	+	+1	+1	0	0	1	+	0	0	0	0	0	0	0	0	0	0
20	++++	0	0	+	+	++	0	0	1	++	0	0	0	0	0	0	0	0	0	0
24	++++	0	0	0	+	+++	0	0	1	+++	1	0	0	0	1	0	0	0	0	0
27	++++	0	0	0	+	++++	0	0	1	++++	1	0	0	1	+	0	0	0	0	0
30	++++	0	0	0	+	++++	0	0	1	++++	+1	0	0	1	+	1	0	0	0	1
48	++++	+++	++++	++++	++++	++++	+++	++R	++	++++	++++	0	0	0	+++	+++	0	0	0	++R
55	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	+++	+++	++++	0	0	0	++R
72	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	+++	+++	++++	0	++	++	++++

G. = growth; 0 = no growth; R = granular growth.

1, +, +1, ++, +++, ++++ = variations in turbidity from minimum to maximum growth (1,000,000,000 per cc.).

TABLE VI.

Colony Types on Agar Transfers from Broth and Asparagine Cultures Containing Bacteriophage.

Broth Cultures.

Incubation	Culture dilution 1:10					Culture dilution 1:100					Culture dilution 1:10,000					Culture dilution 1:100,000				
	Control	Phage 1:10	1:100	1:100,000	1:10,000,000	Control	Phage 1:10	1:100	1:100,000	1:10,000,000	Control	Phage 1:10	1:100	1:100,000	1:10,000,000	Control	Phage 1:10	1:100	1:100,000	1:10,000,000
	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.
hrs.																				
7	S++++	0	0	0	0	S++++	0	0	0	S+	–	–	–	–	–	–	–	–	–	–
30	S++++	S++++	S+++ R+	S+++ R+	S+++ M+	S++++	S++ R+ M+	S++ R+ M+	E++++	E++++	S++++	0	0	R++++	S+ R+ M++	S++++	0	0	R++ M++	0
48	S++++	S++ R++	S++ R++	S+ R+++	S+ R++ M+	S++++	S++++	S++ R++	E++++	E++++	S++++	0	0	R++++	S++ M++	S++++	0	0	R++++	S+ M+++
72	S++++	S++ R++	S++ R++	S+ R+++	S+ R+++	S++++	R+++ M+	S++ M++	S+++ R+	S++ R++	S++++	0	0	S+ R+++	S+++ R+	S++++	0	0	S+ R+++	M++++

Asparagine Cultures.

7	S++++	0	0	E++++	S++++	S++++	0	0	S+++ R+	S++ R++	–	–	–	–	–	–	–	–	–	–
30	S++++	S+++ R+	S+++ R+	S+++ R+	S+++ R+	S++++	S++ R++	S++ R++	S++ R++	S+++ R+	S++++	0	0	0	0	0	0	0	S+++ R+	0
48	S++++	S++++	S++++	S+++ R+	S+++ R+	S++++	S++++	S++++	S++ R++	S++ R++	S++++	0	0	S++++	E++++	S++++	0	0	R++++	S++ R++
72	S++++	S++++	S++++	S+++ R+	S++++	S++++	S+++ R+	S+++ R+	S++ R++	S++ R++	S++++	0	0	S+++ R+	S++ R++	S++++	E+	E++++	S+++ R+	S++ R++

C. = colonies; S = smooth colony (possibly includes SR colonies); M = mucoid-resistant colony; R = rough-resistant colony; E = smooth phage-eaten colony; – = no agar transfer.

0 = no colonies; + = 1 – 10 colonies; ++ = 11 – 20 colonies; +++ = 21 – 50 colonies; ++++ = 50 + colonies.

Experiment 6.—This experiment was planned to test the effect of growth in bacteriophage-containing broth on colony formation.

Two fluid media were used, beef infusion broth, pH 7.4, and a modification of Uchinsky's asparagine solution.[4] The test was run in duplicate in each medium. The single cell Strain 3 was employed. 17 hour broth and asparagine cultures from the stock agar slant were diluted in the corresponding media to make final bacterial concentrations of 5×10^{-1}, 10^{-2}, 10^{-4}, and 10^{-5}. Five cultures of each dilution were made; one served as a control and the other four received bacteriophage in dilutions of 6×10^{-1}, 10^{-2}, 10^{-5}, and 10^{-7}. The tubes were incubated at 37°. At short intervals thereafter the amount of growth was estimated in terms of turbidity and a loop of the culture was transferred to 15 cm., plain agar plates. These in turn were incubated and examined at 24 and 48 hours for number and types of colonies present. The findings are summarized in Tables V and VI.

In the broth series the control tube, inoculated with about 100,000,000 organisms, dilution 1:10, showed growth at 4 hours and maximum turbidity at 12, while tubes containing bacteriophage showed no definite growth for 12 hours, and did not reach a maximum until 27 hours. Tubes receiving an inoculation of 10,000,000 organisms, dilution 1:100, appeared quite similar, except that growth began later in all tubes. The control tube containing 100,000 organisms, dilution 1:10,000, showed abundant growth at 12 hours; similar tubes with high concentration of bacteriophage, 6×10^{-1} and 10^{-2}, remained sterile, while those in which bacteriophage was present in smaller amounts, 6×10^{-5} and 10^{-7}, showed growth at 20 hours and maximum turbidity at 30 hours. The control tube inoculated with 10,000 organisms, dilution 1:100,000 showed growth at 12 hours, which became maximum at 24 hours; those receiving high concentrations of bacteriophage remained sterile, and those in which the amount was low were clear at 30 hours and of maximum turbidity at 48 and 55 hours.

Rough colonies appeared in greatest numbers on plates from tubes inoculated with high concentrations of bacteriophage. Mucoid-resistant colonies were found chiefly on plates from tubes inoculated

[4] The composition of the synthetic medium was as follows: Asparagine, 0.4 per cent; ammonium lactate, 0.6 per cent; sodium phosphate, 0.2 per cent, and sodium chloride, 0.6 per cent in distilled water.

with small amounts of bacteriophage. No variants occurred on the plates from plain broth tubes. The asparagine media series gave similar results, except that growth was slower in all tubes and mucoid-

TABLE VII.

Passage of Variant Colony Forms on Bacteriophage-Containing Agar and Plain Agar.

Date	Passage	Smooth-resistant strains. Passage on 10 per cent phage agar at 37°			Passage	Mucoid-resistant strains. Passage on 10 per cent phage agar at 23°			Passage	Rough-resistant strains. Passage on 10 per cent phage agar at 37°	
		No. 1	No. 3	No. 4		No. 1	No. 3	No. 4		No. 3	No. 4
1927											
2/14	1	SR	SR. MR	SR. MR	1	MR	MR	MR			
15	2	"	"	"	2	"	"	"			
17	3	"	"	"	3	"	"	"			
19	4	"	"	"	4	"	"	"			
21	5	"	"	"	5	"	"	"	1	E.RR	E.RR
23	6	"	"	"	6	"	"	"	2	" "	" "
25	7	"	"	"	7	"	"	"	3	"	"
28	8	"	"	"	8	"	"	"	4	"	"
30									5	"	"
		Passage on plain agar 37°				Passage on plain agar 23°				Passage on plain agar 37°	
2/14	1	SS	SS	SS	1	MR	MR	MR			
15	2	"	" RR	"	2	"	"	"			
17	3	"	"	"	3	"	"	"			
19	4	"	" RR	"	4	"	"	"			
21	5	"	"	"	5	"	"	"	1	RR	RR
23	6	"		"	6	"	"	"	2	"	"
25	7	"		"	7	"	"	"	3	"	SS
28	8	"		"	8	"	"	"	4	SS	"
28	8	—		—	8	SS*	SS*	SS*			
30									5	"	"

SS = smooth-susceptible colony; SR = smooth-resistant colony; MR = mucoid-resistant colony; RR = rough-resistant colony; E = wart-like, "eaten" colony.

* Duplicate passage at 37°.

resistant colonies were not obtained. In four other similar experiments, however, both mucoid-resistant and rough-resistant colonies were recovered.

Briefly, then, bacteriophage added to nutrient media stimulates

the transformation of smooth colony types to mucoid and rough. Solid media and low temperature favor the mucoid forms; fluid media usually favors the development of rough types. These mucoid and rough colony forms differ from those obtained in plain media (Experiments 1, 2, 3) in that they resist the lytic action of bacteriophage.

The stability of these colony variants was then tested by growing them on plain and bacteriophage-containing media. The following experiment shows the result of passage on solid media with or without the addition of bacteriophage.

Experiment 7.—Cultures from the stock agar slant were streaked directly over the surfaces of plain and phage-containing agar plates. These were incubated at 37° and 23°C. Two single colonies of each variant type resulting were then selected and passed for a number of times on plain and bacteriophage-containing agar. Two well isolated colonies were chosen for each transfer. The results of these passages are shown in Table VII.

Three strains of smooth-resistant variants (SR) remained type-pure for eight passages on 10 per cent phage-containing agar, but on plain agar reverted almost immediately to the original smooth-susceptible colony form. The appearance of a few rough colonies on the second and fourth passages of Strain 3 is consistent with the results obtained in Experiment 4. Three mucoid-resistant variants (MR) remained constant for eight passages on 10 per cent phage agar at 23°, and on plain agar as well. But such variants grown on plain agar at 37° revert quickly to the smooth-susceptible form. Two rough-resistant variants passed on 10 per cent phage agar became small and wart-like; passed on plain agar, they reverted to the smooth form after two or three passages. Some rough variants, however, have remained constant for as many as fourteen passages before reverting to smooth.

Hence, the tests indicate that smooth-resistant, mucoid-resistant, and rough-resistant colony cultures, grown on bacteriophage-containing agar, tend to remain type-pure, but when placed on plain agar, become transformed to the original smooth type colony form.

Passage of variant forms in fluid media was carried out with a number of single cell strains from smooth-resistant, mucoid-resistant, and rough-resistant variant colonies. Each culture was transferred

from stock agar to plain broth and broth containing varying amounts of bacteriophage. After 24 hours incubation, a loop of plain broth culture was transferred to a fresh tube of broth, and a loop of bacteriophage-broth culture to a fresh tube of bacteriophage broth. At the same time subcultures were made on plain agar plates. These were incubated and examined at 24 and 48 hours.

When passed in plain broth for more than two or three transfers, single cell cultures from rough-resistant and mucoid-resistant colonies streaked on plain agar plates at 37° showed smooth-susceptible colonies. Smooth-resistant colony cultures reverted to smooth-susceptible after six or seven broth transfers. Bacteriophage added to the broth in a ratio of 10 per cent or more tended to inhibit the reversion phenomenon and maintain the variant cultures type-pure. Smooth-resistant strains remained relatively constant, mucoid-resistant forms remained so at 23° and became smooth-resistant at 37°; rough-resistant cultures usually showed some smooth reverted forms when grown at 37° and mucoid-resistant forms at 23°. Thus, in fluid culture, the variant colony strains were found to revert quickly to the original smooth-susceptible type, unless bacteriophage in large quantity were present and continually added to the cultures.

DISCUSSION AND SUMMARY.

The experiments described in this paper are part of a larger number which we have been carrying out for the past 2 years. Although they have not entirely fulfilled their purpose, which was to explain the so called "mutation," "dissociation," "Umwandlung," "transformation" process, they have served to formulate a helpful working hypothesis.

Certain difficulties inherent in this kind of study must be recognized. One is that colony formation is a property associated with growth on solid media and any procedure involving the use of fluid media introduces a change which is relatively uncontrolled. Another is that mucoid and rough colony forms are not the only variant types encountered; at best, they may be considered as being the most frequent. Finally, it is apparent to us that the findings reported in this paper with respect to enteritidis organisms do not correspond with those of other species of organisms, especially of the respiratory

group which we have studied (1, *b*). Possibly, however, they apply generally to the typhoid, paratyphoid-enteritidis species.

To summarize, it may be stated that the transformation process in the mouse typhoid enteritidis group is an easily reversible one, controlled in part, at least, by three factors, any of which may conceivably operate under natural conditions: (1) temperature, which, influences the appearance of mucoid forms; (2) fluidity of culture media, which tends to favor rough variants; and (3) bacteriophage, which stimulates the appearance of both variants. Since by manipulating these factors the transformation process may be incited at will in either direction, it is probably not genetic in nature.

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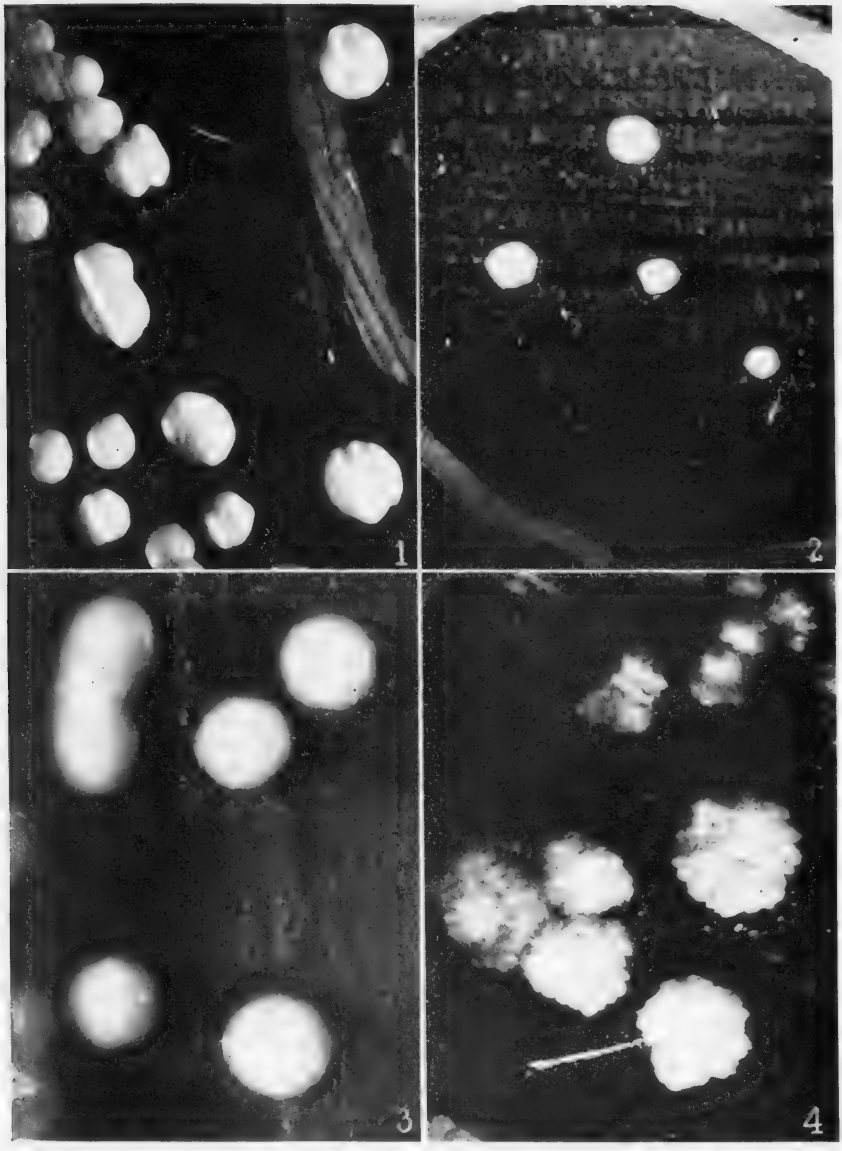
EXPLANATION OF PLATE 33.

FIG. 1. Smooth-susceptible colonies of *B. enteritidis*.
FIG. 2. Smooth-resistant colonies of *B. enteritidis*.
FIG. 3. Mucoid-resistant colonies of *B. enteritidis*.
FIG. 4. Rough-resistant colonies of *B. enteritidis*.



(Webster and Burn: Mouse typhoid infection. II.)

STUDIES ON THE MODE OF SPREAD OF B. ENTERITIDIS MOUSE TYPHOID INFECTION.

III. Studies of Bacterial Cells Taken from Smooth, Mucoid, and Rough Colonies.

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The experiments described in the previous paper (1, *a*) indicate that in general the so called colony "mutation," "Umwandlung," "dissociation," or "transformation" process, as it occurs in mouse strains of *B. enteritidis*, is not of a genetic nature, but is a reversible phenomenon, influenced by at least three factors: temperature, fluidity of medium, and presence of specific bacteriophage. Further knowledge of the nature of the transformation process was sought by making a comparison of the bacterial cells contained in the smooth and the variant colonies.

Appearance of Individual Cells during Early States of Multiplication.

At the outset, single bacilli were examined during early multiplication in order to detect morphological differences in individual young cells taken from smooth, mucoid, and rough colonies.

Reimann's technique (2) was employed. 2 or 3 drops of melted agar were placed on a sterile, hollow ground slide and allowed to cool. Subsequently a small loop of a suspension of cells from a given colony, diluted in sterile 0.85 per cent salt, was placed on the surface of the agar. The dilution was so adjusted that a loop contained 10 to 20 bacterial cells. The preparation was then placed on a microscope stand and examined in a warm box with a dry objective at a magnification of 690 diameters. Aseptic precautions were maintained throughout.

Single cells from smooth-susceptible, smooth-resistant, mucoid-susceptible, mucoid-resistant, and rough-resistant colonies were fol-

lowed through the 2, 4, 8, 16, and 32 cell stages. Many strains were used, but no differences in cell morphology or behavior were detected. In so far as we could determine, young bacilli from the various colony types were indistinguishable.[1]

TABLE I.

Growth of Smooth and Variant Strains of B. enteritidis in Broth.

Incubation time	Average bacterial count per cc.					
	1/3/26 Smooth-susceptible	1/3/26 Mucoid-susceptible	1/3/26 Rough-resistant	1/6/26 Smooth-resistant	1/6/26 Mucoid-resistant	1/6/26 Rough-resistant
hrs.						
0	12	9	5	11	14	11
1	21	18	8	8	18	12
2	65	32	22	31	30	45
3	216	88	127	178	245	191
4	1870	560	430	975	1190	1540
5	8600	3040	3473	6380	11,093	9800
6	52,533	13,000	10,000	35,400	59,600	75,533
7	380,000	140,000	290,000	288,700	443,000	582,000
8	2,850,000	390,000	595,000	1,260,000	3,347,000	4,300,000
9	16,033,333	4,250,000	2,000,000	9,133,333	18,900,000	15,800,000
10	63,533,333	19,270,000	216,000,000	43,300,000	92,533,333	135,933,333
24–26	940,000,000	820,000,000	920,000,000	1,085,000,000	1,470,000,000	1,370,000,000
days						
103–110	2,113,000	48,200	3,520,000	1,920,000	0	334,000
286	—	—	—	0	0	0

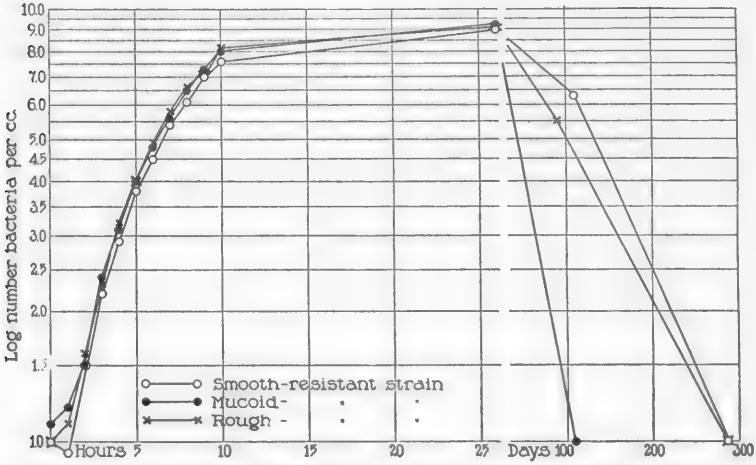
Dark-field illumination and staining methods likewise failed to exhibit any distinguishing characteristics.

[1] A somewhat similar study of smooth and rough cells has been made by Nutt (3). She found that "rough cultures showed the typical bending type of division, producing at the end of the day, irregular masses with projecting angles. In the smooth cultures, the sliding divisions gave rise to the smooth, even masses typical of the smooth colony." Apparently, the emulsions used for seeding in these observations were not washed, but contained the metabolic products which accumulate during cell growth. It is not impossible, therefore, that unwashed cells do, at the outset, behave differently, whereas washed cells behave similarly until metabolic substances accumulate.

Rate of Multiplication of Cells from Smooth and Variant Colonies.

Tests were then made of the growth rates of single cell bacterial cultures obtained from smooth and variant colonies.

Experiment 1.—Variant colonies of Strain 3 were obtained from a single cell smooth-susceptible colony strain by methods outlined in the preceding paper (1, *a*). From these colonies, single cell cultures were procured, according to Reimann's technique (2). These variant strains were transferred to stock agar slants and tested carefully for type purity before use.



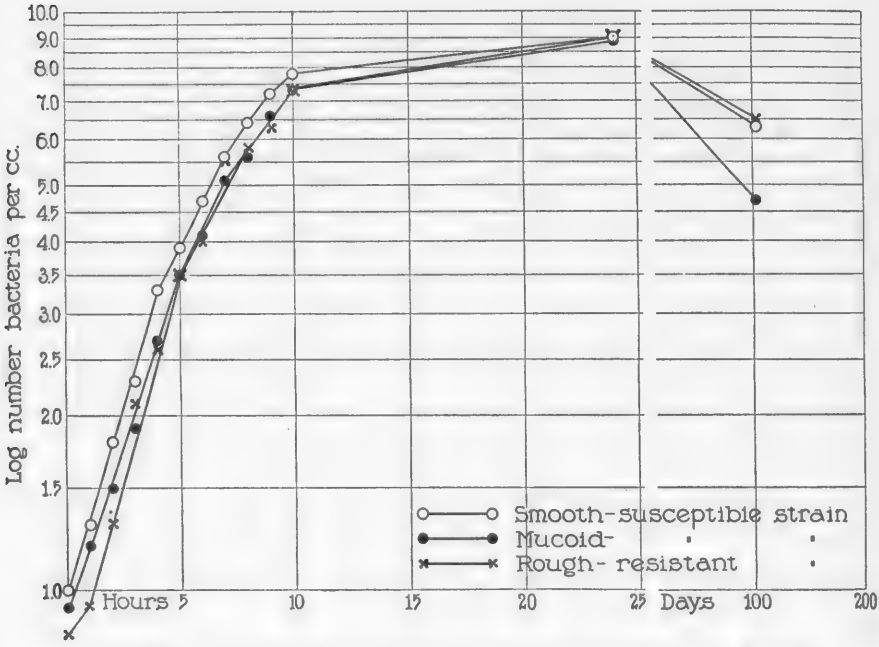
TEXT-FIG. 1. Growth of single cell cultures of *B. enteritidis* in broth. Smooth-resistant, mucoid-resistant, and rough-resistant strains.

18 hour broth cultures from the stock single cell smooth and variant strains were diluted in broth to 1/1,000,000. 1.5 cc. of this dilution was added to 150 cc. of broth in Erlenmeyer flasks, at 37°. Counts were made immediately, and thereafter at hourly intervals, for 10 hours. Further counts were made at 24 hours, and 100 and 280 days. The plating method was employed for counting, and care taken to insure the type purity of each strain. The results of this experiment are shown in Table I and Text-figs. 1 and 2.[2]

[2] These observations were made by Dr. I. W. Pritchett.

The figures (Table I) and curves (Text-figs. 1 and 2) indicate that the growth rates of single cell strains from smooth, mucoid, and rough type colonies are essentially identical.

Thus it appears that in morphology, gross behavior, and growth rate, young cells, 1 to 10 hours old, from any of the several colony



TEXT-FIG. 2. Growth of single cell cultures of *B. enteritidis* in broth. Smooth-susceptible, mucoid-susceptible, and rough-resistant strains.

types studied, are indistinguishable. The next tests were planned to compare certain fundamental properties of mature cells 18 to 24 hours old, taken from different type colonies.

Rate of Migration in an Electric Field.

Cataphoresis measurements were undertaken to compare the potentials of single cell bacilli obtained from smooth and variant colonies.

The Northrop type of cataphoresis cell was employed and standardized according to his methods (4). Readings were taken at 3/16, 7/16, and 11/16 of the cell depth, using both directions of current. The current was regulated to give a potential difference in the cell of 5 volts per cm. From the formula,

$$\frac{\frac{\mu\ (83.33)}{\text{No. of sec.}}}{5} \times 14,$$

the potential in millivolts was calculated. 18 hour growths of single cell cultures were used, and in the tests reported, washed four times in distilled water and suspended in Northrop's buffer (5).

TABLE II.

Rate of Migration of Cultures from Smooth and Variant Colonies.

Test	Source of culture									
	SS		SR		MS		MR		RR	
	sec.	*mv.*	*sec.*	*mv.*	*sec.*	*mv.*	*sec.*	*mv.*	*sec.*	*mv.*
1	5.2	45.2	—	—	5.1	45.7	5.1 5.0	45.7 46.7	—	—
2	5.6	41.8	5.7	40.9	—	—	—	—	5.9	39.5

SS = single cell culture from smooth-susceptible colony; SR = single cell culture from smooth-resistant colony; MS = single cell culture from mucoid-susceptible colony; MR = single cell culture from mucoid-resistant colony; RR = single cell culture from rough-resistant colony.

Table II shows the results of two tests. The migration rate and calculated potential of cells from different colonies were found to be similar. Other tests on unwashed cells and washed cells suspended in distilled water, 0.85 per cent NaCl, or 1 per cent phosphate affected the migration rate in the usual manner (6), but brought out no differences among the various cultures employed.

Oxygen Absorption (Determinations by Dr. Hawkins).

An attempt was made to compare the respiration rate of single cell cultures from smooth and variant colonies by measuring the amount of oxygen absorbed by each under conditions of equilibrium.

Technique.—Single cell cultures from smooth and variant colonies were grown in broth 18 to 24 hours, and washed four times in Ringer's solution containing 0.025 mols of sodium bicarbonate per liter. Final suspensions were then made in Ringer-bicarbonate solution in such a way as to contain relatively the same number of bacilli per cc. Bacterial counts were made before and after the tests to determine the actual numbers of living organisms in each suspension. The cultures were then given to Dr. James Hawkins who determined the amount of oxygen absorbed by each culture.[3]

TABLE III.

Type	No. of bacteria in millions per cc. of suspension	C.mm. of O_2 used per hr. per 100,000,000 of bacteria
SS	71	2.3
	45	2.3
	200	1.6
SR	55	2.7
	426	1.7
	174	2.6
RR	80	1.9
	727	1.0
	125	1.8
MR	278	1.3
	218	2.6
	245	2.8
MS	310	1.2
	400	1.3
	140	2.7

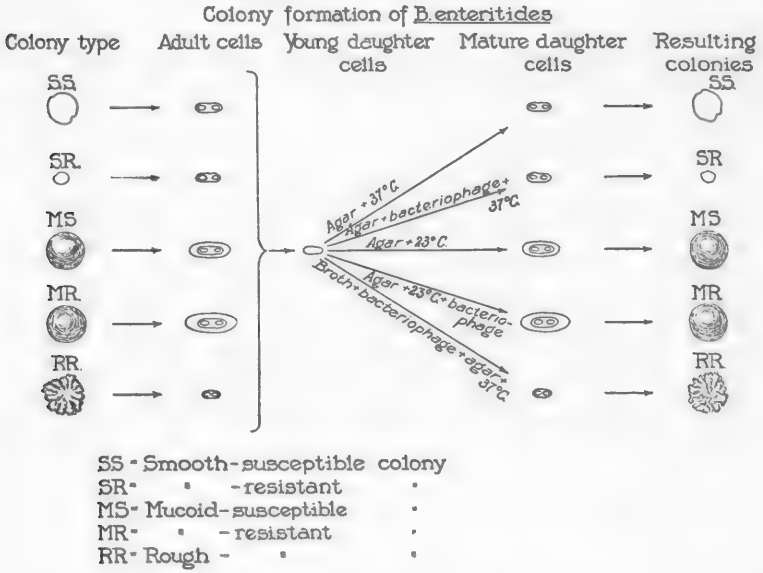
SS = cells from smooth-susceptible colony; SR = cells from smooth-resistant colony; MS = cells from mucoid-susceptible colony; MR = cells from mucoid-resistant colony; RR = cells from rough-resistant colony.

The hydrogen ion concentration was adjusted to pH 7.6 by bubbling through the suspension a mixture of 5 per cent carbon dioxide and 95 per cent oxygen. 8 cc.

[3] Full descriptions of Dr. Hawkin's technique are to be found in the following papers: Warburg, O., and Minami, S., *Klin. Woch.*, 1923, ii, 776. Warburg, O., Negelein, E., and Posener, K., *Klin. Woch.*, 1924, iii, 1062. Warburg, O., *Biochem. Z.*, 1923, cxlii, 317. Minami, S., *Biochem. Z.*, 1923, cxlii, 334. Warburg, O., *Biochem. Z.*, 1924, clii, 51. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309. Murphy, Jas. B., and Hawkins, J. A., *J. Gen. Physiol.*, 1925, viii, 115.

and 3 cc. portions of the suspension were measured accurately into a Warburg type of cell. 5 cc. of this same Ringer's solution, without bacteria, was placed in another cell to act as a control for change in temperature and barometric pressure. The cells were attached to Barcroft manometers, and after the residual air in the cells and manometers had been swept out by the oxygen-carbon dioxide mixture, they were sealed. The manometers were placed on a mechanical shaker with the cells immersed in a constant temperature bath at 37.5° ± 0.01°C. and shaken. The manometers, with the cells immersed in the constant temperature bath, were left for 15 minutes to allow them to come to equilibrium and were then read every 15 minutes.

The amount of oxygen consumed by the bacteria is determined in c.mm. per hour. The results are shown in Table III.



TEXT-FIG. 3. Diagram showing theory of colony formation of *B. enteritidis*.

These figures indicate that the oxygen absorption rate per unit of time per cell in suspensions from the typical smooth-susceptible colonies and their mucoid and rough variants is approximately the same. Hence, the rate of respiration, a fundamental cell property, may be considered uniform for the cells of the different type colonies.

The various observations thus far made were designed to bring out fundamental differences between bacilli from smooth and variant colonies. That they failed to do so, in that no differences in (1)

morphology, or (2) growth rate were noted in young cells, and no differences in (3) potential, or (4) in respiration rate in mature cells, does not prove that young bacilli from each of the several colony types are essentially the same. However, the tests do point in that direction, and hence confirm the theory stated in the preceding paper. Accordingly, one may consider colony morphology as the gross appearance of a population of adult microbes fundamentally similar but modified by certain environmental circumstances, rather than as the expression of any special genetic characters common and specific to each individual in the population. Such a view would predicate the genetic uniformity of young daughter cells derived from individuals of all the colony types, and would explain colony modifications as representative of acquired properties occurring during the lifetime of the individual bacilli, in response to certain definite environmental conditions (Text-fig. 3).

Staining Characteristics.

Notwithstanding this, cells from 18 to 24 hour growths do differ in certain properties according to the colony type from which they originated. One of these differences is in staining quality. Bacilli from the mucoid-susceptible or resistant colonies appear swollen and stain poorly. Capsules may be demonstrated. Bacilli from the rough-resistant colonies appear small and shrunken. The bacterial cytoplasm appears reduced in amount. These differences are seen very clearly when Wright's blood stain is used.

Differential Characteristics of Cells from "Susceptible" and "Resistant" Colonies.

Three other characteristics distinguish cells from *susceptible* and *resistant* colonies: namely, (1) their behavior in the presence of nutrient media containing bacteriophage; (2) their acid agglutination zone, and (3) their virulence.

Cell Behavior in the Presence of Bacteriophage.—Single cell cultures derived from smooth-, mucoid-, and rough-*susceptible* colonies grow poorly or not at all on agar containing 0.1 per cent or more specific bacteriophage, while similar implants from *resistant* colonies, smooth,

mucoid, or rough, multiply luxuriantly and form type-specific resistant colonies. These differences have been discussed at length in the previous paper (1, *a*).

Differences in Acid Agglutination.—The second difference between *resistant* and *susceptible* colony cultures is that the former flocculate in acid buffers at pH 3.8 to 4.2, while the latter remain suspended.

Technique.—18 hour single cell cultures from the stock agar slant were tested for type purity and washed four times in distilled water. 1 cc. of a suitably diluted suspension was then added to 2 cc. of acid solutions of differing pH values. When Na acetate-acetic acid buffers were used, suspensions from the resistant colony types (smooth, mucoid, and rough) flocculated at pH 2.5 to 4.0. In Na lactate-lactic acid buffers, they agglutinated at pH 3.0 to 3.8, and in dilutions of hydrochloric acid at pH 3.8 and 4.0 (7). Suspensions from susceptible colonies (smooth, mucoid, or rough) did not flocculate at any acid dilution employed. $LaCl_3$, however, from dilutions of 1/20 to 1/20,480 agglutinated all cultures alike.

Virulence of Single Cell Cultures from Smooth-Susceptible and Variant Colony Types.—Single cell cultures from bacteriophage-resistant colonies, smooth, mucoid, and rough, proved markedly less virulent than cultures from the smooth-susceptible colony strains. The details of these experiments are contained in the following paper (1, *b*).

Action of Bacteriophage on Living, Non-Multiplying Bacterial Cells.

That the bacilli from various type colonies resemble one another closely in their fundamental characteristics and yet differ in acid agglutination, virulence, and resistance to bacteriophage, according to whether they come from *susceptible* or *resistant* colonies, suggests the possibility that the differences are due to an adsorption of bacteriophage on the surface of the *resistant* bacilli. Accordingly, this phenomenon, and its modifying effects would be considered as the result of acquired, not of inherited, alterations.

Experiments in another paper (1, *b*) support this view entirely. Furthermore, such a conclusion is in harmony with the latest studies on the mechanism of bacteriophage action. D'Hérelle (8), and all late investigators are of the opinion that the first step in bacteriophage action is a surface binding of phage and bacterial cell. Still another series of studies on the mechanism of bacterial agglutination are ex-

plained by considering flocculation as the result of a specific coating of bacteria by globulin (9).

We have sought further information as to (1) whether bacterial cells do adsorb bacteriophage and (2) whether *susceptible* cells in contact with bacteriophage under conditions where the possibility of multiplication is reduced to a minimum, do become altered to resemble cells from *resistant* colonies.

The technique of the tests was so arranged as to reduce as far as possible bacterial multiplication.

18 hour growths of single cell cultures in broth were washed four times in distilled water. 0.8 per cent NaCl gave similar results. The washed cells were then suspended in various buffer solutions, glycocoll (5), and 0.2 and 1 per cent phosphate. When distilled water or 0.8 per cent NaCl was used as the diluent, the pH of the systems dropped rapidly to 6 or 6.6, thus rendering the system unfavorable for the test. 0.2 per cent phosphate was finally employed as a standard.

The number of cells per cc. was estimated by comparison with standardized suspensions and checked by actual counts on dilution plates. The test suspensions with or without phage were then placed in the ice box at 4° from 4 to 144 hours. Preliminary tests and direct counts showed that very few cells were killed during the washing process, or during the first 24 hours in the ice box at 4°C., provided the cells were suspended in a concentration of not more than 10^9 per cc.

Specific bacteriophage was prepared in broth in the usual way and added to the suspensions in as small a volume as possible. Subsequent determination of the amount of bacteriophage remaining in the solution was obtained by throwing down the cells, removing the supernatant, heating it to 60° for 30 minutes, or preferably filtering it through an M Berkefeld candle. Dilutions of the filtrate were then made from unit volume to 10^{-9} in 5 cc. broth, and about 10,000 bacilli of an 18 hour single cell culture of the smooth-susceptible type strain added to each. The amount of growth in each tube at 37° was then recorded at a definite interval of 6 to 10 hours after inoculation.

Cells acted upon by bacteriophage at 4° were tested in several ways for comparison with cells from variant type colonies. After being removed from the ice box, the suspensions were centrifuged and the cells washed. Oxygen absorption rate, potential, and acid agglutination zones were determined, as well as virulence. The results of these several tests are given in Tables IV to VI.

Table IV shows the results of one of a number of such tests with single cell strains from the typical smooth-susceptible colonies. Under these conditions, 200,000,000 organisms per cc. removed all bacterio-

phage from the solutions of 1/1000 and 1/100 concentration. The 10 per cent solution of bacteriophage was not entirely exhausted; the

TABLE IV.

Action of Bacteriophage on Suspensions of Non-Multiplying Bacteria.

Organisms 2,000,000 per cc. 2/16/26	Count per cc. after 4°C., 18 hrs.	Amount of phage remaining in supernatant												Acid agglutination of bacterial sediment							
		Phage control	Dilution of supernatant											Dilution N/10 HCl							
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	Control	1	2	3	4	5	6	7	8
		1/100	0	0	0	0	0	0	0	+	+	+	+	−	−	−	−	−	−	−	−
		1/1000	0	0	0	0	0	0	+	+	+	+	+	−	−	−	−	−	−	−	−
Organisms plus bouillon control (1)	180,000,000	−	−	−	−	−	−	−	−	−	−	−	−	0	0	0	0	0	0	0	0
Organisms plus bouillon control (2)	200,000,000	−	−	−	−	−	−	−	−	−	−	−	−	0	0	0	0	0	0	0	0
Organisms plus phage 1/10	115,000,000	−	0	0	0	+	+	+	+	+	+	+	+	0	0	0	4.0 +	4.2 +	0	0	0
Organisms plus phage 1/100	184,000,000	−	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
Organisms plus phage 1/1000	210,000,000	−	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0

+ = good growth at 8 hours; 0 = no growth at 8 hours; − = no test.

− = no test; + = agglutination at pH 4.0 and 4.2.

supernatant, when filtered, definitely inhibited the growth of the test strain. Organisms in a solution containing phage in a dilution of 1/1000 and 1/100 did not agglutinate, while those in a 10 per cent

TABLE V.

Action of Bacteriophage on Suspensions of Non-Multiplying Living and Dead Bacteria.

Organisms 1,000,000 per cc. 2/28/26	Amount of bacteriophage remaining in supernatant											Acid agglutination of bacterial sediment																					
	Phage control	Dilution of supernatant										Na lactate-lactic acid buffers										N/10 HCl											
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Control	Control	pH 2.4	2.7	3.0	3.3	3.5	3.8	4.1	4.5	4.7	Control	1	2	3	4	5	6	7	8		
	1/10	0	0	0	0	0	0	0	0	+	+	+																					
	1/100	0	0	0	0	0	0	0	+	+	+	+																					
	1/1,000,000	0	0	0	0	+	+	+	+	+	+	+																					
Living plus bouillon control	–	–	–	–	–	–	–	–	–	–	–	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living plus phage 1/10	–	0	0	0	0	0	+	+	+	+	+	+	0	0	0	0	0	C	C	0	0	0	0	0	0	0	0	C	0	0	0	0	0
Living plus phage 1/100	–	0	0	0	+	+	+	+	+	+	+	+	0	0	0	0	0	C	C	0	0	0	0	0	0	0	0	C	0	0	0	0	0
Living plus phage 1/10,000	–	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living plus phage 1/1,000,000	–	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dead plus bouillon control	–	–	–	–	–	–	–	–	–	–	–	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dead plus phage 1/10	–	0	0	0	0	0	0	0	+	+	+	+	0	0	0	0	C	C	C	C	0	0	0	0	0	0	3.8	4.1	0	0	0	0	0
Dead plus phage 1/100	–	0	0	0	0	0	+	+	+	+	+	+	0	0	0	0	C	C	C	C	0	0	0	0	0	0	0	C	C	0	0	0	0
Dead plus phage 1/10,000	–	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dead plus phage 1/1,000,000	–	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

– = no test; + = good growth at 8 hours; 0 = no growth at 8 hours.

0 = no agglutination; C = complete agglutination.

solution flocculated at the same pH, 3.8 to 4.2, as do bacilli from phage-resistant variant colonies. A number of these tests under slightly differing conditions gave similar results.

Subsequently the ratio of organisms to quantity of bacteriophage was varied and the test repeated. The tests indicated that the ratio of number of organisms to amount of bacteriophage taken out of the solution is relatively constant and hence that in all probability, each bacillus takes up a certain and definite amount of the lytic substance.

Table V compares the activity of living and dead bacilli. A single cell culture of smooth-susceptible colony type bacilli was used, washed four times in distilled water, and divided into two portions. One was heated to 75°C. for 1 hour. Evidently dead bacilli take up bacteriophage from the solution, but to a less degree. Probably, however, there exists a definite quantitative relation as in the case of the living organisms. Furthermore, dead bacilli are not agglutinated in acid solution unless placed in contact with the bacteriophage. When so treated, however, they flocculate at pH 3.8 to 4.2, as do living cells of the same sort and cells from bacteriophage-resistant variant colonies.

Oxygen absorption and potential measurements were made on suspensions of washed cells in contact with bacteriophage at 4°C. They were found to take up the same amount of oxygen per unit of time and to migrate at the same rate in the electric field as control suspensions and cells from any of the various type colonies. Hence bacteriophage in contact with living smooth-susceptible cells in the resting state, as well as cells from variant colony types, did not affect these fundamental physiological properties.

Changes in virulence were determined by the usual procedures. Washed cultures in contact with bacteriophage at 4°C. were later washed again and given to mice intraperitoneally and *per os*. Similar suspensions without the addition of phage were used as controls. Not only were the former suspensions of low pathogenicity, but they failed to multiply in the body of the host and disappeared rapidly in a manner precisely similar to single cell cultures from phage-resistant variant colonies. That this was not due to lysis was made certain by the behavior of broth and agar plate transfers. The details of this experiment are described in the following paper (1, *b*).

The fact that cells from smooth-susceptible colonies may be treated with phage under conditions where the possibility of cell multiplication is reduced to a minimum and changed so that they behave in a way precisely similar to cells taken directly from the bacteriophage-

TABLE VI.

Action of Bacteriophage on Suspensions of Non-Multiplying Cells from Bacteriophage-Resistant Colony Types.

Organisms No. per cc.	Amount of bacteriophage remaining in supernatant												Acid agglutination of bacterial sediment Na lactate-lactic acid buffers									
	Phage control	Dilution of supernatant											Hydrogen ion concentration of buffers									
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Control	Control	2.4	2.7	3.0	3.3	3.5	3.8	4.1	4.5	4.7
	1/100	0	0	0	0	0	0	0	0	+	+	+										
	1/1000	0	0	0	0	0	0	0	+	+	+	+										
SR (115,000,000)													0	0	0	0	C	C	C	C	C	0
SR (115,000,000) plus phage 1/100	–	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	C	0
SR (115,000,000) plus phage 1/1000	–	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	C	0
MR (94,000,000)													0	0	0	0	C	C	C	C	C	0
MR (94,000,000) plus phage 1/100	–	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	C	C	C	C	C	0
MR (94,000,000) plus phage 1/1000	–	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0	0	C	C	C	C	0
RR (120,000,000)													0	0	0	0	0	C	C	C	C	0
RR (120,000,000) plus phage 1/100	–	0	0	0	0	0	0	0	+	+	+	+	0	0	0	0	C	C	C	C	C	0
RR (120,000,000) plus phage 1/1000	–	0	0	0	0	0	0	0	+	+	+	+	0	0	0	0	C	C	C	C	C	0
	– = no test; + = good growth at 8 hours; 0 no growth at 8 hours.												0 = no agglutination; C = complete agglutination.									

resistant variant colonies is considered by us as evidence that this type of change is purely an environmental phenomenon, and that the particular bacteriophage action is a surface adsorption process similar to that of agglutinins adhering to the surface of their specific bacterial cell. If this view is correct, one would expect cells from bac-

teriophage-resistant colonies to adsorb no further bacteriophage under similar conditions, and to remain unaltered.

Table VI summarizes the results of a test in which cell suspensions from smooth-, mucoid-, and rough-*resistant* colonies were treated with bacteriophage as in the previous tests. None of them removed any noticeable quantity of phage from the solutions, and none of them showed alterations in acid agglutination zone.

DISCUSSION.

The above experiments are a continuation of those reported in the preceding paper, the purpose of which has been to secure a better understanding of the mechanism of colony transformation and of the part it plays in determining the spread of mouse typhoid. The observations on colony variation, although somewhat irregular, indicate that type of colony depends upon environmental rather than hereditary factors. This point of view is supported by the present studies on bacterial cells taken directly from different type colonies, and by the fact that living bacteria from the typical smooth colonies, placed in contact with bacteriophage under conditions where cell multiplication was restrained, are altered so as to resemble the bacterial cells from the variant bacteriophage-resisting colonies.

To consider the transformation process to mucoid and rough variant colonies as one of environmental degradation, and the reversion to smooth type as mere accumulation of daughter cells in the absence of such environment, does not explain bacterial transformation in the hemorrhagic septicemic group of organisms. Here the process seems non-reversible, and cells from variant colonies are found to differ markedly in fundamental properties (1, *c*). We put forward the view stated merely as a working hypothesis, and as best explaining at the present time the facts at hand relating to the transformation of organisms of the mouse typhoid *B. enteritidis* group.

CONCLUSIONS.

1. During early stages of multiplication, single cells from smooth-, mucoid-, and rough-susceptible and variant colonies show no differences in morphology or growth rate.

2. Cells from 18 to 24 hour single cell cultures of these various colony types possess similar oxygen absorption and cataphoretic migratory rates. In staining property, the cells from mucoid colonies appear larger, and those from rough colonies smaller, than the typical cells from smooth-susceptible colonies.

3. Cells from bacteriophage-resistant colonies differ from those of bacteriophage-susceptible colonies in their ability to multiply luxuriantly in the presence of bacteriophage, and in their tendency to flocculate in acid solutions at pH 3.8 to 4.1, as well as in their low degree of virulence.

4. Cells from smooth bacteriophage-susceptible colonies in contact with bacteriophage under conditions where multiplication is restrained may be altered so as to resemble the cells from the bacteriophage-resistant colonies.

5. These facts furnish evidence that bacteriophage adheres to the surface of the bacterial cell and that the various cell changes and colony alterations are of an environmental rather than genetic nature.

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STUDIES ON THE MODE OF SPREAD OF B. ENTERITIDIS MOUSE TYPHOID INFECTION.

IV. The Relative Virulence of Smooth, Mucoid, and Rough Strains.

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In another paper of the series, we pointed out that when bacteria from typical smooth colonies of mouse strains of *B. enteritidis* are grown under certain environmental conditions, they become transformed into mucoid and rough colony forms (1, *a*). Organisms from these "variant" colonies, especially the smooth, mucoid, and rough bacteriophage-resistant forms, were found to differ in certain respects from those of the original smooth strain (1, *b*). One of the distinguishing characters proved to be loss of virulence, a change which also was effected experimentally by placing smooth type bacteria in contact with bacteriophage. It is proposed now to describe the titrations of virulence carried out and to give details of two additional experiments which partly explain the virulence mechanism involved.

The question of the measurement of bacterial virulence has been referred to at length elsewhere (1, *c*). At this time it is sufficient to emphasize again the necessity of controlling the dosage variable by using known numbers of bacilli, and the host factor by inoculating a sufficient number of mice of one race, similar age and weight, and with no previous exposure to the specific infection. The bacteria should be introduced by way of the normal portal of entry, and the mice should then be placed in separate cages in order to reduce the number of bacilli reingested. Under these conditions the reaction of an adequately large group of mice should be relatively constant, a definite number of individuals dying at a relatively constant rate, others recovering, and still others remaining apparently unaffected.

These effects may be shown graphically by plotting total mortality per cent against time.

The mice employed in the titrations recorded below came from the Rockefeller Institute breeding room. They consisted of an albino strain, inbred for 15 years. At the time of inoculation each animal was 12 to 14 weeks old, and weighed 18 to 20 gm. They are believed to have been unexposed to *B. enteritidis* mouse typhoid, although a very occasional carrier (less than 1 per 1000) has been encountered from time to time among the stock animals (1, *d*). After inoculation by stomach tube, each animal was placed in a separate glass jar in a special room cared for by an experienced attendant.

TABLE I.

Relative Virulence of Smooth, Mucoid, and Rough Strains of B. enteritidis. Experiment 1.

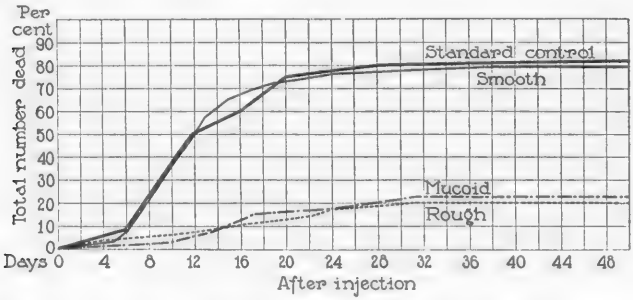
No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures; colony types	Results of fecal cultures			
							9th day		31st day	
		No.	Per cent	No.	Per cent		No. positive	Per cent and type of colonies	No. positive	Per cent and type of colonies
27	SS	22	82	22	82	Smooth 22; mucoid 2	3 (23%)	Smooth 2; mucoid 2	0	0
26	MR	6	23	6	23	Smooth 5; mucoid 5	5 (21%)	Mucoid 5	1 (5%)	Mucoid 1
27	RR	6	22	5	18.5	Smooth 2; rough 5	5 (20%)	Rough 5	2 (9.1%)	Rough 2

SS = culture from smooth-susceptible colony; MR = culture from mucoid-resistant colony; RR = culture from rough-resistant colony.

Experiment 1.—The first test was made with the smooth type stock strain No. 3, obtained in 1922 from a case of spontaneous mouse typhoid (1, *a*, *b*). Its virulence was determined carefully in 1923 and found to be relatively constant and high (1, *c*). Since that time it has been kept in the ice box on an agar slant and transferred at irregular intervals. During October, 1925, the culture was treated with bacteriophage. This, when added to broth or agar cultures of the bacilli, led to the appearance of typical smooth, mucoid, and rough colony variants which were resistant to lysis. The desired variant colonies were transferred to stock agar slants and identified and examined for type purity with a minimum number of subcultures.

Transfers from the stock agar cultures of the original smooth type and the

mucoid and rough variants were made to broth and incubated for 17 hours at 37°. The following day suitable dilutions were made, plates were poured for counting colonies, and ½ cc. of the 1–100 broth dilution of each culture was given *per os* to three series of mice. Each of twenty-seven mice received about 5,000,000 of the original smooth type bacilli; twenty-six mice, about 7,500,000 mucoid variant bacilli, and twenty-seven mice 10,000,000 of the rough type. Each culture employed was found to be type-pure. Mice found dead from day to day during the experiment were autopsied and cultured from heart's blood, spleen, and intestine. The types and relative numbers of organisms were determined. Fecal cultures on all mice 3 days before inoculation were negative. 11 and 31 days after inoculation the surviving mice were cultured to determine the presence of *B. enteritidis* in their feces.



TEXT-FIG. 1. Relative mortality of mice given smooth, mucoid, and rough strains of *B. enteritidis*. Experiment 1.

The protocols of this experiment are summarized in Table I and the specific mortality figures plotted in Text-fig. 1. 82 per cent of the mice receiving organisms of the original smooth type died, all showing pure cultures of smooth colonies in heart's blood, spleen, and intestine, and 9.1 per cent showing besides a few mucoid forms. The mortality rate (82 per cent) corresponded almost exactly to that observed with the same strain 2 years previously (1, *c*). No mouse typhoid bacilli were obtained from the stool cultures of the survivors. 23 per cent of the mice receiving the mucoid strain died. At autopsy one showed the mucoid type only; four mucoid and smooth types, and onethe smooth type alone. Five of the survivors showed mucoid types in their feces 11 days after injection, and only one at 31 days. From the remainder no mouse typhoid bacilli were obtained. 22 per cent

of the rough culture group died, and at autopsy one yielded no mouse typhoid bacilli from heart's blood, spleen, and intestine cultures, three rough forms only, and two both rough and smooth forms. Of the survivors five showed rough forms 11 days after injection, and one at the end of the 31 day period.

From the results of this virulence titration we conclude that the smooth type strain No. 3 of *B. enteritidis* is of relatively high pathogenicity, with exactly the same virulence as 2 years ago, and that its mucoid and rough variants are markedly less virulent. In the tissue of the mouse the smooth culture remained type-pure and virulent, except for the presence of an occasional mucoid colony,[1] while in many instances the mucoid and rough variants tended to revert quickly to the original smooth form.

2 weeks after the first titration a second was made, in order to compare the virulence of reverted and original smooth cultures.

Experiment 2.—Four strains were used—that designated smooth-susceptible No. 1, came from the original stock strain No. 3 and served as the control; smooth-susceptible Nos. 2 and 3 were obtained from smooth colonies found on autopsy plate cultures from two mice in Experiment 1 of the group inoculated with the mucoid culture; and smooth-susceptible No. 4, from a reverted smooth colony obtained from a plating of the mucoid culture used in Experiment 1, after six daily passages in broth.

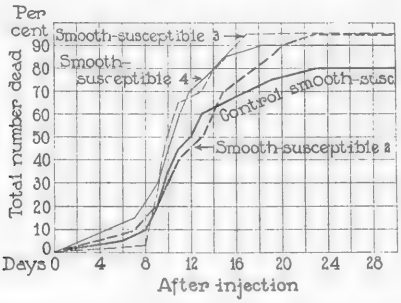
The technique employed for the inoculations was similar to that used in Experiment 1. Twenty mice received 5,000,000 bacilli of the original smooth-susceptible culture No. 1; twenty received 5,000,000 of the reverted smooth-susceptible No. 2 forms; twenty were given 4,500,000 of smooth-susceptible No. 3, and twenty 7,500,000 of smooth-susceptible No. 4 cultures. Each culture was tested carefully for type purity and specific characteristics. As far as could be determined, they were identical.

The specific mortality for each group is plotted in Text-fig. 2. The close similarity in amount and rate of deaths in the various groups indicates that the virulence of the different reverted smooth type cultures was the same as that of the original strain.

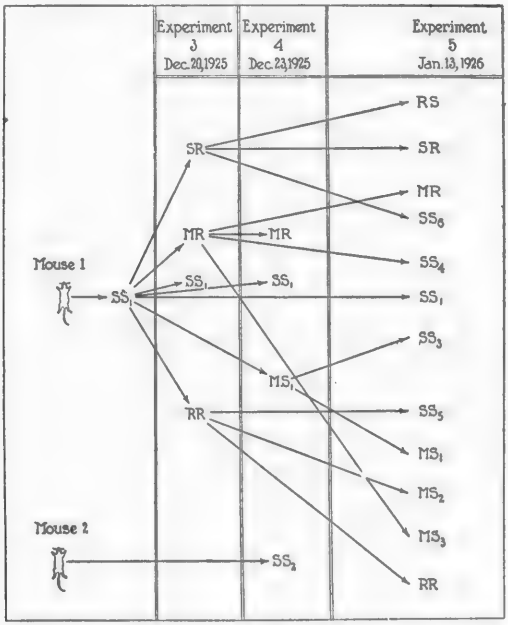
A further titration was made to test the virulence of recently isolated single cell cultures of smooth and variant strains of *B. enteritidis*.

[1] Plates were examined at 24, 48, and 72 hours. The presence of mucoid colonies was attributed to the fact that the plates had been left at 23° after 24 hours incubation at 37° (1, *a*).

Experiment 3.—A mouse from one of the experimental epidemic populations referred to above (1, *d*) was found dead on December 5, 1925. At autopsy, smooth type colonies of *B. enteritidis* were obtained in pure culture from heart's



TEXT-FIG. 2. Relative mortality of mice given smooth and "reverted" smooth strains of *B. enteritidis*. Experiment 2.



TEXT-FIG. 3. Diagram showing source of cultures used in Experiments 3, 4, and 5.

blood, spleen, and intestines. A single cell strain was procured from the heart's blood culture by Reimann's technique (2). A high titre bacteriophage was then obtained by daily passage and filtration of this single cell smooth type strain. This bacteriophage, added to young broth cultures of the single cell smooth type strain, gave rise to mucoid and rough variants resisting lysis. From these variant colonies single cell cultures were obtained, identified serologically, and tested for type purity.

17 hour broth transfers from the stock agar slants of these various single cell cultures (Text-fig. 3) were suitably diluted in broth, plated, and administered by stomach tube in ½ cc. volume to four groups of mice. Each of twenty mice received 5,000,000 of the original smooth-susceptible strain; twenty were given 9,600,000 of the smooth-resistant variant; twenty 5,500,000 of the mucoid-resistant variant, and twenty 5,000,000 of the rough-resistant form. The animals were then placed in separate jars and treated precisely as in Experiments 1 and 2. On the following day the colonies on the dilution plates were examined carefully for type purity and identified serologically. All proved type-specific.

TABLE II.

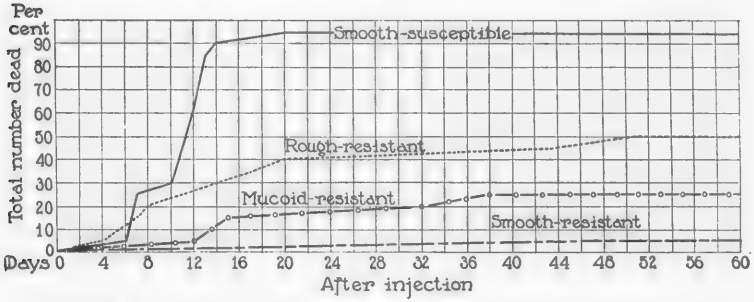
Relative Virulence of Smooth, Mucoid, and Rough Strains of B. enteritidis. Experiment 3.

No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures: colony types
		No.	Per cent	No.	Per cent	
20	SS	19	95	19	100	Smooth 19; mucoid 8
20	SR	8	40	1	5	Mucoid 1
20	MR	15	75	5	25	" 5
20	RR	18	90	9	45	Rough 8; smooth 2

The results are summarized in Table II and specific mortality rates are plotted in Text-fig. 4. 95 per cent of the mice receiving the original smooth-susceptible type died. At autopsy smooth colonies were obtained from each animal and in addition mucoid forms from eight. Eight mice receiving the smooth-resistant variant succumbed; from one a mucoid form was recovered; from the remaining seven no mouse typhoid bacilli were obtained. 75 per cent of the mice given the mucoid-resistant culture died. Of these only 25 per cent showed *B. enteritidis*. Mucoid forms alone were present. 90 per cent of the rough series succumbed—only 45 per cent harbored the specific organisms. From 35 per cent of these, rough colonies alone were cultured; from one, both smooth and rough, and from one other smooth

types alone. Thus, two discrepancies were encountered in this experiment: first, the appearance of mucoid colonies in autopsy cultures of mice which had received smooth forms, and second, the death of a considerable number of mice giving negative autopsy cultures. The first peculiarity is explained by the fact that the autopsy plates were often left at room temperature after 24 hours incubation; the second we are not able to explain at the present time. It is interesting to note, however, that nearly all mice giving negative cultures died within the first 6 days, a period below the usual incubation time.

If specific deaths are plotted with reference to time, as in Text-fig. 3, the results appear quite consistent with those of the first two experiments, and indicate that the recently isolated single cell, smooth-



TEXT-FIG. 4. Relative mortality of mice given smooth, mucoid, and rough strains of *B. enteritidis*. Experiment 3.

susceptible culture is of high virulence, as in the case of those previously titrated, and that the single cell variants—smooth-, rough-, and mucoid-resistants—are relatively of low pathogenicity. Finally, there is further corroborative evidence that in the animal tissues the variant types (in this experiment, the rough form) revert to the smooth-susceptible type.

On December 23, 3 days after the last series was inoculated, a fourth titration of the same and other variant cultures was carried out.

Experiment 4.—The following strains were employed (see Text-fig. 3): SS 1, the original single cell, smooth-susceptible strain used in Experiment 3; SS 2, a single cell, smooth-susceptible culture similar in every respect to SS 1, obtained at the same time from another mouse in the epidemic series; MR 1, the same

single cell, mucoid-resistant variant from SS 1 used in Experiment 3, and MS 1, a single cell, mucoid-susceptible variant, obtained from an agar plate culture of the original SS 1, after three daily broth passages. Each culture was given to twenty mice—7,600,000 to each mouse in the SS 1 group; 5,900,000 to each in the SS 2 group, and 5,000,000 to each in the MS 1 and MR 1 groups. Tests for identification and type purity observations and autopsies were made precisely as in the previous experiments. All colonies on the dilution plates were type-pure and specific. The results are shown in Table III and Text-fig. 5.

90 per cent of the mice receiving the original SS 1 culture died, and showed at autopsy smooth-susceptible colonies in the heart's blood and spleen culture plates. In two cases besides, a few mucoid colonies were present. 95 per cent of the SS 2 series died: 85 per cent with

TABLE III.

Relative Virulence of Smooth and Mucoid Strains of B. enteritidis.

Experiment 4.

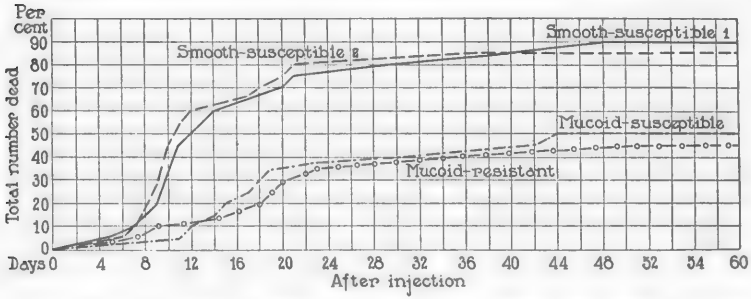
No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures: colony types
		No.	Per cent	No.	Per cent	
20	SS-1	18	90	18	90	Smooth 18; mucoid 2
20	SS-2	19	95	17	85	" 17
20	MS	14	70	10	50	Mucoid 10
20	MR	11	55	9	45	" 9

smooth-susceptible colonies in heart's blood and spleen cultures, and 10 per cent with no typhoid organisms whatsoever. 70 per cent of the MS series died; from 50 per cent type-specific organisms were recovered; from four, no mouse typhoid bacilli. 55 per cent of the MR series succumbed; 40 per cent with mucoid-resistant colonies in the autopsy cultures, one mouse with mucoid-susceptible forms, and two with no typhoid colonies. From a comparison of the curves in Text-fig. 4, which include only mice dying with positive typhoid cultures, it is evident that the two single cell, smooth-susceptible strains are of similar high pathogenicity, and that the single cell, mucoid-susceptible and mucoid-resistant variants are of comparatively low virulence. Little evidence of type reversion *in vivo* was obtained in this experiment.

A final titration was made with the original single cell, smooth-

susceptible Strain SS 1, used in Experiments 3 and 4, together with several of its variants and reverted forms.

Experiment 5.—The cultures were designated as follows (see Text-fig. 3): SS 1, MS 1, MR 1, SR 1, and RR 1, used in Experiments 3 and 4, and SS 3, SS 4, SS 5, SS 6, MS 2, MS 3, and RS 1, reverted forms derived from the above named cultures by repeated broth passage and plating. SS 3, a reverted, single cell, smooth-susceptible culture came from the mucoid-susceptible variant (MS 1); SS 4, a single colony, smooth-susceptible culture from the mucoid-resistant MR strain; SS 5, a single cell, smooth-susceptible culture from the rough-resistant RR variant, and SS 6, a single colony, smooth-susceptible culture from the smooth-resistant (SR) strain. MS 2, a single cell, mucoid-susceptible culture, came from the rough-resistant RR strain; MS 3, a single colony, mucoid-susceptible



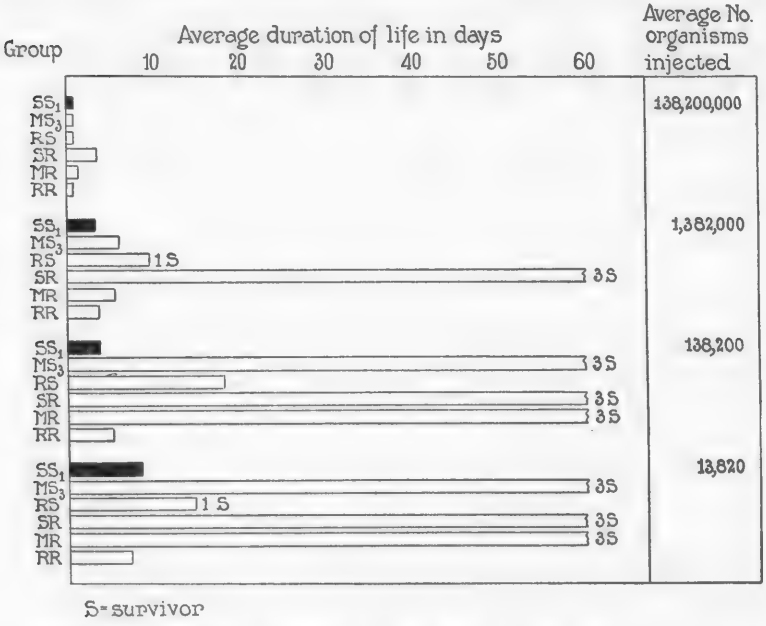
TEXT-FIG. 5. Relative mortality of mice given smooth and mucoid strains of *B. enteritidis*. Experiment 4.

culture, from the mucoid-resistant MR strain, and RS, a single cell, rough-susceptible culture, from the smooth-resistant SR strain.

On January 13, 1926, 17 hour broth cultures from the stock agar slants were diluted in broth, plated, and given *per os* to twelve series of mice. Each series consisted of twenty animals obtained from the same source, and cared for in the same way as those used in the previous experiments. The dosage per mouse of each culture was as follows: SS 1, 9,000,000; SS 3, 7,500,000; SS 4, 9,000,000; SS 5, 9,000,000; SS 6, 8,875,000; MS 1, 7,250,000; MS 2, 14,000,000; MS 3, 5,000,000; RS 1, 4,250,000; SR 1, 7,625,000; MR 1, 6,750,000; and RR 1, 8,885,000. The SS 1, MS 3, SR 1, MR 1, RR 1, and RS 1 strains were also injected intraperitoneally into similar mice. Each strain was administered to twelve mice. Three received a dilution of 10^{-1}; three, 10^{-3}; three, 10^{-4}; and three, 10^{-5}. The actual dosage in numbers of bacilli was calculated from counts of the dilution plates.

On the following day the plates were tested for type specificity and purity. The mice placed in individual jars were observed for a period of 2 months, and those dying were autopsied and cultures were made from the spleen, heart's blood, and intestines. The types and relative numbers of *B. enteritidis* colonies were determined by inspection, sugar fermentation, agglutinability in specific sera, and lysis in bacteriophage-containing broth.

The dilution plates of each culture, after 24 hours incubation, showed type-specific and pure colonies. Text-fig. 6 shows the results



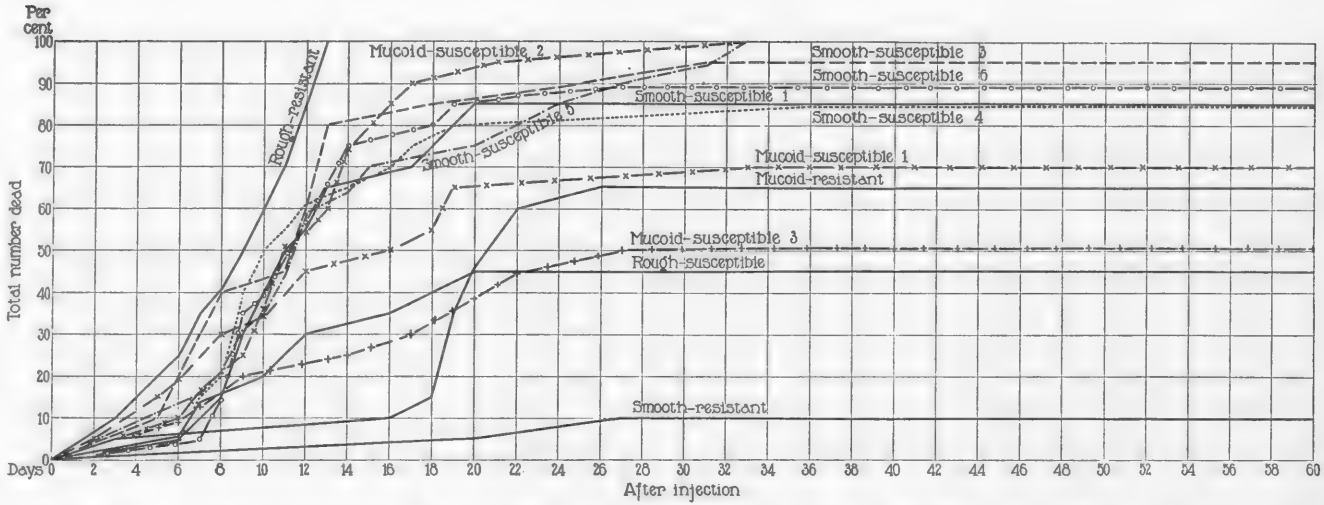
TEXT-FIG. 6. Relative mortality of mice given smooth, mucoid, and rough strains of *B. enteritidis*. Intraperitoneal titrations. Experiment 5.

of the intraperitoneal titrations. A dose of about 138,000,000 of each culture, except the SR 1, killed in 1 day—the mice given this SR 1 strain showed an average survival time of $3\frac{1}{2}$ days. A dose of about 1,382,000 killed the three mice given the SS 1 culture in an average time of 3 days. Those given the RR 1, MR 1, and MS 1 cultures survived on an average of 3, 5, and 6 days, respectively.

The three mice receiving the SR 1 culture in this dilution survived. A dose of about 138,200 bacilli killed the SS 1 group in an average time of 4 days; the RR 1 group in 5 days, and the RS 1 group in 19 days. Those receiving the MS 1, MR 1, and SR 1 cultures survived. The average surviving time of mice receiving 13,820 of the SS 1 bacilli was 8 days; that of a similar dose of the RR 1 bacilli, 7 days, and of the RS 1 bacilli, 15 days. This dosage of MS 1, SR 1, and MR 1 was not fatal. The titration indicates, therefore, that the SS 1 culture, when injected intraperitoneally into mice, is highly virulent, and that its variants, with the exception of the RR 1 strain, are of low pathogenicity.

At autopsy mice receiving the RR cultures showed, instead of RR colonies, the original smooth-susceptible forms in large numbers. Mice from the other groups showed only type-specific colonies.

The results of the *per os* inoculations are shown in Text-fig. 7, and the autopsy protocols in Table IV. The total specific mortality of the various SS cultures varied between 85 and 100 per cent; that of the MS cultures between 50 and 100 per cent; of the RS cultures 45 per cent; the SR cultures 10 per cent; the MR cultures 65 per cent; and the RR cultures 100 per cent. 85 per cent of the mice receiving the SS 1 culture succumbed and showed on agar plate cultures from the various organisms typical smooth colonies of *B. enteritidis*. 90 per cent of the mice receiving the SS 3 culture succumbed. All of these animals showed smooth-susceptible colonies on the cultures taken at autopsy. 85 per cent of the SS 4 group died. From these smooth-susceptible colonies alone were obtained. 100 per cent of the SS 5 group died. Smooth-susceptible colonies were recovered from all. 90 per cent of the SS 6 group succumbed. From these smooth-susceptible colonies were obtained. Thus it is evident that the reverted, smooth-susceptible forms coming from the phage-resistant, smooth, mucoid, and rough variants are similar in virulence to the original smooth-susceptible strain. 90 per cent of the mice receiving the MS 1 culture died. Of these 70 per cent showed *B. enteritidis* cultures at autopsy. Fourteen of the latter showed mucoid-susceptible colonies in pure culture, or in large numbers. From two, smooth-susceptible forms were likewise obtained. All mice receiving the MS 2 culture died and showed on the agar cultures taken from various organs



TEXT-FIG. 7. Relative mortality of mice given smooth, mucoid, and rough strains of *B. enteritidis*. *Per os* titrations. Experiment 5.

large numbers of mucoid-susceptible colonies. From four animals, however, smooth-susceptible forms were cultured. 75 per cent of the MS 3 series died. Of these 50 per cent showed *B. enteritidis* colonies. In nine mucoid-susceptible forms were obtained, and in two smooth-susceptible forms. The effect of the mucoid-susceptible cultures, therefore, appears less severe than that of the smooth-susceptible forms, and colony type reversion from mucoid- to smooth-susceptible noteworthy. 75 per cent of the mice receiving the RS culture succumbed. From 45 per cent rough-susceptible colonies were obtained in pure

TABLE IV.

Relative Virulence of Smooth, Mucoid, and Rough Strains of B. enteritidis. Experiment 5.

No. of mice injected	Colony type of culture injected	Mortality		"Specific" deaths		Results of autopsy cultures: colony types
		No.	Per cent	No.	Per cent	
20	SS-1	17	85	17	85	Smooth 17
20	SS-3	18	90	18	90	" 18
20	SS-4	17	85	17	85	" 17
20	SS-5	20	100	20	100	" 20
20	SS-6	18	90	18	90	" 18
20	MS-1	18	90	14	70	Mucoid 14; smooth 2
20	MS-2	20	100	20	100	" 20; " 4
20	MS-3	15	75	10	50	" 9; " 2
20	RS	15	75	9	45	Rough 9
20	SR	7	35	2	10	Smooth-resistant 2
20	MR	17	85	13	65	Mucoid-resistant 12; smooth-resistant 4
20	RR	20	100	20	100	Smooth 20

culture. Seven of the smooth-resistant group died, two of which showed pure cultures of smooth-resistant *B. enteritidis* colonies. 85 per cent of the mucoid-resistant group died, 65 per cent with positive cultures of *B. enteritidis*. From these latter mucoid-resistant colonies were found in twelve, and smooth-resistant in two. All the mice receiving the rough-resistant culture died, and from each animal smooth-susceptible forms were obtained in pure culture. Thus it is seen that the rough-susceptible and mucoid-resistant forms are of comparatively low virulence, and that the smooth-resistant form is per-

haps of lowest pathogenicity. The fact that the rough-resistant culture reverted to smooth-susceptible in the tissues of the mice makes this particular titration of no value, except to show the comparative instability of this colony type.

The results of the several titrations (Experiments 1 to 5) bring out the following facts: (1) The virulence of type-pure, smooth-susceptible cultures was similar and relatively high. Single cell strains fresh from mice, or preserved at 4°C. for more than 2 years, or recovered from so called "reverted variants" after broth or mouse

TABLE V.

Intraperitoneal Injections of Bacteria from Smooth-Susceptible and Smooth-Resistant Colonies.

Experiment 6.

After injection	Group receiving smooth-susceptible organisms						Group receiving smooth-resistant organisms					
	Mouse 1	2	3	4	5	6	1	2	3	4	5	6
hrs.												
1	+++	+++	+++	+++	++++	+++	0	0	0	0	+	0
2	+++	+++	+++	+++	++	++++		0	0	0	0	0
4	++	+++	∞	∞	++++	∞	0	++	0	0	0	0
6	++++	++	+	+++	++	∞	0	0	0	0	0	0
24	0	+++	0	+++	++	∞	0	0	0	0	0	0
48		Dead				Dead						
72			Dead	Dead	Dead							
96	Dead						S	S	S	S	S	S

+ = 1–10 colonies per drop; ++ = 10–20 colonies per drop; +++ = 20–50 colonies per drop; ++++ = 100 colonies per drop; ∞ = 100+ colonies per drop.

S = surviving and well.

passage, behaved essentially alike. Under the conditions of the experiments, little colony type variation of the smooth-susceptible type occurred *in vivo*. (2) The mucoid-susceptible variant proved somewhat less virulent and tended to revert to the original smooth-susceptible form. The rough-susceptible and mucoid-resistant variants behaved in essentially the same manner. (3) The smooth-resistant forms were of very low virulence, and more stable in the tissues of the mouse than the other variants. (4) Finally, the rough-resistant variant, when

type-pure, was likewise of relatively low virulence, but showed a marked tendency *in vivo* to revert to the original smooth-susceptible form.

The titrations described above indicate that the greatest differences in virulence occur between strains from bacteriophage-susceptible and bacteriophage-resistant colonies. An attempt was made to determine the cause of the reduced virulence on the part of the strains from bacteriophage-resistant colonies.

Experiment 6.—Single cell cultures of smooth-susceptible and smooth-resistant strains of No. 1 were taken from the stock slant and grown 18 hours in broth. Each was then diluted 1:1000 in broth and 1 cc. given intraperitoneally to six mice. This dosage determined by the dilution method proved to be 850,000 organisms of the smooth-susceptible culture and 1,250,000 of the smooth-resistant. Immediately after injection, and at 1, 2, 4, 6, and 24 hours thereafter, each mouse's peritoneal cavity was aspirated by means of a short, beveled No. 24 syringe needle, and 1 drop of the exudate spread over the surface of agar plates. The number of colonies resulting and the fate of the two groups of mice are shown in Table V.

1 hour after injection, all mice receiving the smooth-susceptible culture showed more than 100 colonies per drop on the plates. One mouse of the smooth-resistant series showed about ten colonies; the rest were negative. Later the bacteria increased rapidly in the exudate of the first group, while in the smooth-resistant group, cultures remained sterile. All smooth-susceptible mice were dead within 3 days; those receiving the smooth-resistant organisms survived and appeared in good health at the end of 3 weeks.

This test shows that the smooth-susceptible bacteria multiplied in the peritoneal cavity of the host and that the smooth-resistant forms did not. In fact, other tests indicated that within ½ hour after injection the smooth-resistant organisms had practically disappeared from the peritoneal exudate. Hence, one may infer that the same events occur when the bacilli are given by the normal portal of entry, and may interpret the negative stool cultures and negative autopsy cultures of the preceding titrations as being due to the fact that the variant type bacteria did not survive in the intestinal tract or the tissues of the host.

A final experiment was planned to test the possibility of altering the

virulence of smooth-susceptible cells by merely placing them in contact with bacteriophage.

Experiment 7.—The preliminary control tests were carried out as described in the previous paper.[2] A single cell culture of smooth-susceptible strain No. 1 was grown 18 hours in broth, washed four times in distilled water, and resuspended in 0.2 per cent dibasic sodium phosphate. A uniform suspension was made to contain about 500,000,000 per cc. 27 cc. was placed in contact with 3 cc. concentrated bacteriophage in broth, 27 cc. in another flask with 3 cc.

TABLE VI.

Titration of Bacteriophage Present after Contact with Smooth-Susceptible Cells at 4°C.

Suspension	Dilution of suspension										
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Control
Bacteria plus broth.....	0	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Bacteria plus bacteriophage.....	0	0	0	0	0	0	+	+++	+++	+++	+++

0 = no growth at 6 hours; + = slight growth at 6 hours; ++ = moderate growth at 6 hours; +++ abundant growth at 6 hours.

TABLE VII.

Acid Agglutination of Organisms in Contact with Bacteriophage at 4°C.

Suspension	pH 2.4	3.01	3.18	3.53	4.03	4.25	4.65	5.01	5.31	Control
Organisms plus broth.....	0	0	0	0	0	0	0	0	0	0
Organisms plus bacteriophage.....	0	0	0	++	++	0	0	0	0	0

of broth, and in a third flask, 27 cc. of phosphate was added to 3 cc. of bacteriophage. The three flasks were then kept at 4°C. for 16 hours. Bacterial counts before and after this treatment showed that no multiplication had occurred.

The two suspensions of bacteria plus bacteriophage and broth were then centrifuged and the supernatant filtered and tested, together with the phosphate-phage control for the presence of bacteriophage. The sedimented organisms were

[2] Webster and Burn, Paper III, p. 880.

TABLE VIII.

Virulence Titration of Smooth-Susceptible Organisms in Contact with Bacteriophage at 4°C.

Mouse group	Results of aspiration 2 hrs. after injection	Results of aspiration 24 hrs. after injection	Duration of life
			days
10 mice: organisms plus broth; dose 12,000,000	+++, +++, +++, +++, +++, +++, +++, +++, +++, +++	∞, ∞, ∞, ∞, ∞, ∞, ∞, ∞, ∞, ∞	3, 3, 3, 3, 3, 3, 3, 4, 4, 4
10 mice: organisms plus broth; dose 120,000	+++, +++, +++, +++, +++, +++, +++, +++, +++, +++	+++, +++, +++, +++, +++, ∞, ∞, ∞, ++, ++	4, 4, 5, 5, 5, 6, 6, 7, 7, 7
10 mice: organisms plus bacteriophage; dose 10,000,000	++, ++, ++, ++, ++, ++, ++, ++, ++, ++	+++, 0, 0, 0, 0, 0, 0, 0, 0, 0	3
10 mice: organisms plus bacteriophage; dose 100,000	++, +, +, +, +, +, +, 0, 0, 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	

0 = no organisms per drop; + = 1–10 organisms per drop; ++ = 10–20 organisms per drop; +++ = 20–50 organisms per drop; ∞ = 50+ organisms per drop.

washed four times in distilled water, tested for acid agglutinability in buffers, for lysis in broth, and injected intraperitoneally into mice. Ten mice were given 12,000,000 of the smooth-susceptible bacteriophage organisms, and ten 120,000; ten received 10,000,000 smooth-susceptible control organisms, and ten 100,000. Peritoneal aspirations were made at 2 and 24 hours. The results are shown in Tables VI, VII, and VIII.

The titre of the bacteriophage-phosphate control proved to be 10^{-7}, while that of the supernatant in contact with the smooth-susceptible organisms was 10^{-1} (Table VI). Thus, there was evidence that bacteriophage had been adsorbed by the smooth-susceptible cells at 4°C. (previous paper). These cells agglutinated at pH 3.5 to 4.03 in acid buffers, while the control suspension did not (previous paper) (Table VII). Finally, aspirations made from mice receiving the smooth-susceptible broth control cultures showed that multiplication *in vivo* had occurred, while those from mice receiving the smooth-susceptible bacteriophage cultures showed no multiplication, and after 24 hours no survival of the bacteria. Only one of these latter mice died, while all of the former, 10,000,000 group, died within 4 days, and the 100,000 group in 7 days (Table VIII). Suspensions transferred to broth did not undergo lysis; probably, therefore, they did not lyse *in vivo*.

This experiment indicates, therefore, that bacteriophage, presumably by coating the surface of smooth-susceptible cells, prevents multiplication in the tissues of the animal and therefore renders the organisms practically non-virulent. The *per os* inoculation tests, in which it was found that the bacteriophage-resistant variant colony strains given by the normal portal of entry did not survive within the organism, are probably to be explained in the same way.

DISCUSSION.

The virulence titrations of *B. enteritidis* described in this paper have been rigidly controlled; in most instances, single cell strains were used. They show definitely that cultures from mucoid and rough bacteriophage-resistant colonies are less virulent than those from the typical smooth-susceptible colonies. The results agree with those of Arkwright (3), Topley (4), Bronfenbrenner, Muckenfuss, and Korb (5), Jordan (6), Goyle (7), and others who have studied the paratyphoid-enteritidis group, but are distinctly at variance with d'Hérelle's (8)

statements that cultures from resistant colonies are of greater pathogenicity than those from the typical smooth type colony. Furthermore, the tests indicate that type-pure cultures from the common smooth colonies are of similar virulence, whether obtained from mice in epidemic or endemic periods, or from stock agar slants kept for 2 years at 4°C. In this respect, the results agree in general with those of Smith and Nelson (9) as well as with our own earlier work with mouse paratyphoid (*B. pestis caviæ*) (1, *b*), and rabbit pasteurella organisms (*Bact. lepisepticum*) (1, *e*). Topley and his associates (10), on the other hand, consider that the virulence of smooth type cultures of mouse typhoid bacilli fluctuates to a significant degree. Finally, we have shown that the loss of virulence of variant strains from smooth-, mucoid-, and rough-resistant colonies is due to the fact that they do not multiply in the tissues of the host, and we have suggested the possibility that this inability to grow is due to a coating of the bacilli by bacteriophage.

The data contained in three papers of this series (II, III, IV) show clearly that the colony type transformation from smooth to mucoid and rough is reversible; accordingly they agree with the results of Jordan (6). It must be noted, however, that in this respect the intestinal group of bacteria appears to differ from bacteria of the respiratory tract. For example, *Bact. lepisepticum*, *B. friedländeri*, *Bact. diphtheriæ*, and streptococci change from smooth to rough, but as yet no demonstration of the reverse transformation from rough to smooth has been made. The nature of the change is not known; in the case of *B. enteritidis* mouse typhoid organisms, it seems to be acquired rather than hereditary; while the transformations of the respiratory tract organisms mentioned above appear relatively permanent. However, the term "mutation" is not justifiable (d'Hérelle), nor does the "life-cycle" theory (Hadley) find support in our own experimental observations. At present, therefore, two general types of transformation may be recognized: the relatively permanent changes associated with organisms of the respiratory tract and the easily reversible changes of intestinal tract bacteria, where temperature, characteristics of medium, and presence of bacteriophage appear to determine the process. A final and complete explanation, however, must await further experimental observations.

The importance of bacterial transformations in determining the amount and severity of disease is, we believe, relatively slight. And this despite the following facts: (1) that variants of low virulence may be derived experimentally under conditions similar to those prevailing in nature and that they revert again to the original virulent forms; (2) that some of these variants are known to exist "naturally;" and (3) that these variants may possibly revert in nature to the forms of high pathogenicity. Far more significant is the fact that these variant strains are neither common nor abundant in nature and that the smooth type colony is generally known to prevail during all phases of disease, endemic and epidemic. Our own studies of experimentally induced epidemics are confirming this belief amply and demonstrating besides that the virulence of these common smooth type colony cultures is at all times relatively constant.

CONCLUSIONS.

1. Single cell mouse strains of *B. enteritidis* from smooth-susceptible colonies were, under the various conditions tested, of the same high degree of virulence. 2 year old cultures, strains obtained both in interepidemic and epidemic periods, and "reverted" strains from variant colonies showed the same degree of pathogenicity.

2. Single cell strains from variant smooth, mucoid, and rough phage-resistant colonies were definitely less virulent than the usual smooth-susceptible strains. The loss of virulence in each instance resulted apparently from contact with bacteriophage which rendered the individual cells incapable of multiplication in the animal tissues.

3. A reduction of virulence was induced experimentally when washed cultures of the highly pathogenic smooth-susceptible cells were exposed to bacteriophage and thus rendered incapable of multiplication in the inoculated animals.

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STUDIES ON BACTERIAL ENZYMES.

VI. The Maltase of the Diphtheria Bacillus.

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INTRODUCTION.

It is important to obtain knowledge of the mechanism of maltose fermentation by the diphtheria bacillus since the test for acid production from this sugar is one of the means employed in distinguishing the true etiologic agent of diphtheria from certain other morphologically similar but non-pathogenic bacilli. Previous demonstrations (1–6) of the carbohydrate-hydrolyzing enzymes of pneumococci, meningococci, *botulinus* and Welch bacilli (1–6) have proved that the activity of these enzymes is independent of the presence of the living or formed bacterial cell, and at the same time have furnished indirect evidence that these particular bacteria do not attack higher carbohydrates without preliminary hydrolysis. However, since different species of bacteria may form entirely different products from the fermentation of the same sugar (4), it is desirable to obtain experimental evidence of the carbohydrate-hydrolyzing enzymes of important species of bacteria rather than to assume their presence from the simple observation of acid or gas production by living cultures.

In the present investigation, experiments were made to determine whether or not sterile solutions of the intracellular substances of diphtheria bacilli possess the property of hydrolyzing maltose. The bacterial solutions were filtered through Berkefeld candles after physical disintegration of the bacilli by repeated freezing and thawing, and hence were devoid of cell fragments as well as free from living bacteria.

EXPERIMENTAL.

Methods.

Preparation of Diphtheria Bacillus Enzyme Solutions.—The enzyme solution was prepared from a virulent and recently isolated strain of diphtheria bacilli. The procedure was essentially the same as that employed in the preparation of solutions of other bacteria (meningococci, pneumococci, Welch and *botulinus* bacilli) reported in previous papers of this series (3–6).

6 liters of an 18 hour broth culture were centrifuged, and concentrated suspensions of the bacilli were prepared by resuspending the bacterial sediment from each 250 cc. of culture in a volume of approximately 5 cc. Preliminary experiments proved that diphtheria bacilli were much more resistant to disintegration by freezing and thawing than were any of the different bacteria from which enzyme solutions had been prepared in the previous studies. In view of the difficulty of breaking up the bacilli, an attempt was made to facilitate the process by suspending the bacteria in concentrated salt solution. The bacterial suspension was divided into three portions, and the different lots were frozen and thawed under the following conditions: (1) bacilli suspended in the broth supernatant of the original culture, (2) bacilli suspended in the broth supernatant plus 10 per cent NaCl, (3) bacilli suspended in 10 per cent salt solution without broth. It was necessary to continue the freezing and thawing over a long period but after the process had been repeated between 300 and 400 times, stained films revealed a considerable degree of morphological disintegration and a large amount of amorphous, Gram-negative, bacterial substance. Samples of the suspension of disintegrated bacilli were then centrifuged at high speed and the supernatant tested for dissolved bacterial protein by boiling, in order to avoid the danger of filtering the solution before sufficient bacterial dissolution had occurred. When the tests of the supernatant fluids showed the presence of coagulable protein, the entire lot of the bacterial suspension was centrifuged at high speed for 1 hour to remove most of the undissolved bacterial cells and detritus before filtering; the centrifuged supernatants were then passed through a sterile Berkefeld filter.

The filtered bacterial solutions were perfectly clear and were indistinguishable in appearance from the medium in which the bacilli had originally been suspended (broth and salt solution, respectively). The presence of a considerable amount of dissolved bacterial protein derived from the disintegrated diphtheria bacilli was indicated by the heavy precipitation of coagulated protein when the solutions were boiled. So far as could be detected either by this test or by microscopic examination of the stained films, the addition of 10 per cent salt did not facilitate the dis upture of the bacillary bodies. But the boiling test showed that the solutions prepared from the bacteria suspended in broth contained more dissolved protein than did the solution prepared from the bacteria suspended in salt solution alone. Apparently, the presence of broth either facilitates the dissolution of the bacterial cells or renders the liberated bacterial substances more

soluble, for it has been our experience with other bacteria (4–6) that the most active and potent preparations are obtained when broth is used as the suspension medium.

Sterility Controls.—No antiseptics were used; the sterility of each enzyme-substrate mixture was controlled by cultural methods.

Substrates.—20 per cent solutions of the test sugars were boiled for 20 minutes, and then added aseptically to sterile 0.1 M phosphate solution (pH 6.9) in amount sufficient to give a final concentration of 4.0 per cent. The final sugar solutions were distributed into sterile Pyrex tubes for use in the enzyme experiments.

Test of Disaccharide-Hydrolyzing Activity of Sterile Solutions of Diphtheria Bacterial Substances.

Living cultures of the strain from which the sterile bacterial solution was prepared, possessed the usual (7) carbohydrate-fermenting properties of diphtheria bacilli, fermenting the common hexoses and maltose, but not forming detectable amounts of acid from either sucrose or lactose. In the following experiment, tests were made to determine whether or not the sterile solution prepared from the intracellular substances of the diphtheria bacilli possessed the property of hydrolyzing maltose. Tests were included with sucrose as representing a disaccharide not fermented by living diphtheria bacilli and also with lactose as representing a disaccharide which sometimes gives equivocal results in fermentation tests with some strains of the living bacilli (7). Since living cultures of diphtheria bacilli produce acid from glucose and from the other hexoses yielded in the hydrolysis of the disaccharides, tests were also made to determine whether or not the sterile enzyme solution possessed the property of acid formation from hexoses.

Hydrolysis Mixtures.—2 cc. portions of sterile, 4 per cent solutions of maltose, lactose and sucrose were distributed into three series of sterile Pyrex test-tubes, and 1.0 cc. of the sterile diphtheria bacterial solution was added to one tube of each of the substrates. A second series was prepared to serve as controls of the heat lability of the hydrolyzing enzymes by adding the same amount of heat-inactivated (boiled) bacterial extract to each substrate. All of the mixtures were incubated at 37°C. for 72 hours and then tested for the presence of hexoses.

Detection of the Hexose Products of the Enzyme Action.—The sucrose series were tested for reducing sugars with Benedict's solution. The lactose and maltose hydrolysis mixtures (where the substrates themselves are reducing sugars) were tested for hexoses by the biological method described in detail in preceding

papers. The method is based upon the acid fermentation of the hexoses by bacteria which do not ferment the disaccharide from which the hexoses are derived. 1 cc. of each of the test, and of the control hydrolysis mixtures, was put into a series of sterile Pyrex test-tubes; 1 cc. of a suspension of bacteria which produce acid from hexoses but which do not attack the test disaccharide was then added to the different tubes. (An atypical strain of colon bacilli which does not attack maltose was added to the maltose tests; typhoid bacilli which do not attack lactose or sucrose were used for the lactose and sucrose tests.)

TABLE I.

Action of Diphtheria Bacillus Enzyme Solution upon Maltose, Lactose and Sucrose.

Disaccharide hydrolysis by diphtheria bacillus enzymes				Action of diphtheria bacillus enzymes on hexose products
Hydrolysis mixture		pH after action of bacteria used as fermenting agents	Change in pH due to fermentation of hexoses previously formed by diphtheria bacillus enzymes	Acid production
		pH	*ΔpH**	
Maltose	Active diphtheria bacterial solution	6.5	1.1	0
	Heat-inactivated bacterial solution	7.6	0.0	0
Sucrose	Active diphtheria bacterial solution	7.6	0.0	0
	Heat-inactivated bacterial solution	7.6	0.0	0
Lactose	Active diphtheria bacterial solution	7.6	0.0	0
	Heat-inactivated bacterial solution	7.6	0.0	0

* ΔpH indicates change (decrease) in pH.

The "fermentation mixtures" (equal portions of hydrolysis test mixture plus the bacterial-fermenting agent) were shaken, incubated in the water bath at 37°C. for 1½ hours and then centrifuged at high speed to remove the bacteria. Colorimetric determinations of the pH of the clear supernatant fluids were made to detect the production of acid. That the changes in reaction of the fermentation mixtures were limited to the acid produced from the hexoses previously formed by the active diphtheria bacillus enzyme was controlled by the tests on the control series of heat-inactivated enzyme plus the test disaccharides.

Experiments were made with the three different lots of diphtheria bacterial solution (*i.e.* solutions prepared from the bacilli suspended in broth alone, from bacilli suspended in broth plus 10 per cent NaCl and from bacilli suspended in 10 per cent NaCl without broth). Only quantitative differences were obtained in the experiments with the different types of bacterial solution, and the results from the tests with the enzyme solution prepared from the bacilli suspended in broth alone are summarized in Table I.

The results of these experiments (Table I) show that the sterile solution of the diphtheria bacterial substances contained an active maltase which is heat-labile. In contrast to the results in the tests of the maltose-enzyme mixtures, no acid was formed by the hexose-fermenting bacteria when added to the previously incubated mixtures of diphtheria bacillus enzymes and lactose or sucrose solution; this fact is evidence that the sterile bacterial solution contained only maltase and no lactase or sucrase. Thus, the carbohydrate-hydrolyzing activity of the solution prepared from the cellular substances of diphtheria bacilli is parallel to the fermenting activity of cultures of the living bacteria, maltose being the only disaccharide hydrolyzed by the bacterial solution just as maltose is the only disaccharide from which living diphtheria bacilli can produce acid. These results indicate that the acid fermentation of higher carbohydrates by diphtheria bacilli depends upon the presence of a hydrolyzing enzyme to split the specific carbohydrate to hexose constituents.

It is also shown in Table I that the diphtheria bacillus enzyme solution which hydrolyzes maltose, is devoid of the property of forming acid from the hexose products of hydrolysis. Living diphtheria bacteria actively ferment glucose and the failure of the sterile bacterial solution to possess the acid-forming property of the living bacilli is in contrast to its retention of the original carbohydrate-hydrolyzing activity of the living bacteria. The retention of the carbohydrate-hydrolyzing activity by sterile solutions of the bacterial substances of diphtheria bacilli and the loss of the hexose-fermenting activity, is the same phenomenon which has been evident in the similar studies of pneumococci, meningococci, Welch and *botulinus* bacilli (3–6). While the activity of the hydrolyzing enzymes is apparently quite independent of the presence of the living or formed bacterial cell, the production of acid from hexoses seems to be more intimately associated with the morphological integrity of the bacteria.

Endocellular Nature of the Maltase of the Diphtheria Bacillus.

The previous demonstration of the diphtheria bacillus maltase was made with solutions of the bacterial substances prepared from the bacilli suspended in salt solution alone, as well as with solutions prepared from bacilli suspended in the supernatant fluid of broth cultures. Since the bacterial extracts prepared from the bacilli suspended in salt solution contained none of the exocellular substances liberated in the culture medium during growth, the presence of an active maltase in this type of bacterial solution is in itself evidence that the maltase is an intracellular derivative of the diphtheria bacillus. In order to show more definitely that the maltase is not also an exocellular product set free during growth of the bacilli, comparative tests were made of the maltose-hydrolyzing activity of filtrates of broth cultures and of solutions of the intracellular substances.

These tests were made by the same procedure as described for the previous experiment. The results showed that while the solution of intracellular substances of the bacilli hydrolyzed maltose, the filtrate of the young broth culture which contained only extracellular products was devoid of maltose-hydrolyzing activity. Thus, the maltase of the diphtheria bacillus is an endocellular constituent of the diphtheria bacillus, agreeing in respect to its intracellular nature with the carbohydrate-hydrolyzing enzymes of meningococci, pneumococci, *botulinus* and Welch bacilli (1–6).

COMMENT.

The preceding experiments have demonstrated that diphtheria bacilli possess a maltose-splitting enzyme which remains operative in sterile, filtered solutions of the intracellular substances which can be liberated by physical disintegration of the bacterial cells. Since it is of endocellular origin, the maltase represents a heat-labile constituent of the diphtheria bacillus cell. The demonstration of the active maltose-hydrolyzing enzyme is of interest from two different points of view.

First, experimental proof of the maltase adds to the knowledge of the mechanism of carbohydrate fermentation by the diphtheria bacillus. The fact that the sterile bacterial solution contains a maltase but

no lactase nor sucrase is in agreement with the fact that the living bacilli ferment maltose but no other disaccharide, and indicates that the production of acid from disaccharides by diphtheria bacilli depends upon the possession of a hydrolase to split the specific disaccharide to its fermentable hexose constituents. Living diphtheria bacilli actively ferment glucose but the sterile bacterial solution although it hydrolyzes maltose is devoid of the property of forming acid from the glucose products. These relations are analogous to those previously found (1–6) with pneumococci, meningococci, Welch and *botulinus* bacilli, and indicate that the fermentation of maltose by the living diphtheria bacillus involves two separate sets of reactions: (1) hydrolysis, (2) fermentation of, or acid production from, the hexose products; only the first reaction being independent of the presence of the living or formed bacterial cell.

Maltase is one of the most labile of the known constituents of bacterial cells and would be inactivated if drastic methods were employed to disintegrate the cells. Hence the fact that maltase can be obtained in an active state in the sterile solutions of the intracellular substances of diphtheria bacilli is also of interest as proof of the possibility of preparing solutions or extracts of the endocellular constituents of these bacteria without loss of their original activity. In the preparation of the sterile solutions of diphtheria bacilli reported in this paper the bacterial cells were disrupted simply by repeated freezing and thawing, which would be expected to yield the intracellular substances in more nearly the original condition in which they exist in the living bacilli than in preparations obtained by dissolving the bacteria in alkali. However, in order to obtain active preparations containing the desired amount of dissolved bacterial substance (protein) it is necessary to repeat the freezing and thawing process 300 to 400 times, since diphtheria bacilli are much more resistant to disintegration by freezing and thawing than are the bacteria studied in previous investigations (1–6). Xerosis bacilli are even more resistant to this treatment than are diphtheria bacilli, and we have been unable to obtain satisfactory solutions of their intracellular substances by freezing and thawing suspensions of these bacteria. The diphtheria bacillus solutions contain sufficient dissolved bacterial protein to be used in immunological studies, and are being employed for that purpose in investigations to be reported in later papers.

SUMMARY.

Diphtheria bacilli possess a heat-labile, endocellular maltase, which retains its activity in sterile solutions of the intracellular substances which are liberated by physical disintegration of the bacterial cells. The proof of the maltase activity and the detection of the hexose products constitute experimental evidence that the living diphtheria bacillus attacks maltose by way of a preliminary hydrolysis.

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STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY.

IV. The Inheritance of Susceptibility to Tar-Induced Tumors in the Lungs of Mice.

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Previous communications from this laboratory have provided evidence in favor of the theory that susceptibility to tumors of the mammary gland (1) and tumors of the lung (2) is inherited. This evidence is based upon observations of spontaneous tumors. The discovery and perfection of methods of inducing tumors of various kinds have demonstrated the important rôle played by chronic irritation in the origin of neoplasms. It is conceivable that the so called "spontaneous" tumors also are the result of chronic irritation and that they seem to be spontaneous simply because the chronic irritant is unknown to the observer. This is a possible point of view though by no means one necessarily consequent upon the facts at hand. Whatever the relationship between the two kinds of tumors will ultimately prove to be, it has seemed pertinent to investigate the relation between chronic irritation and a demonstrated hereditary susceptibility.

The material used for this purpose consists of two strains of mice chosen from among a number which have been under observation in this laboratory for some years. They are an agouti strain, No. 1194, and the Bagg albinos. A somewhat detailed account of their history and numbers has already been published (2). The Bagg strain has a low rate of mammary gland tumors but a higher rate of lung tumors, while the opposite condition obtains in Strain 1194, which has a higher rate of mammary gland tumors than the preceding group but a low rate of tumors of the lung. The lung tumor rates have been shown (2) to be significantly different in the two stocks. Among 208 mice belonging to Strain 1194 which lived to be at least 12 months old, 6.73 per cent $\pm$ 1.17 had primary neoplasms in the lung. In the Bagg strain 37.04 per cent $\pm$ 2.80, in a total of 135 mice, had

tumors. The difference between the rates is 30.31 ± 3.04 and is undoubtedly significant. A few tumors of other types also have been found, and Strain 1194 produces a small number of epitheliomas of the jaw, but neither strain has developed tumors elsewhere in the skin.

An initial attempt in the investigation of the problem was made by testing the two strains in regard to their reaction to tar painting on the skin (3). The well known method of applying tar extract periodically to the skin between the shoulders was used, and in many cases carcinomas resulted. The percentage of tumors and the rates at which they appeared were practically the same in the two groups. Although the strains differed in respect to their spontaneous tumor rates, they showed no difference in their response to tar painting on the skin. It was pointed out at the time that the spontaneous tumors originated in one type of epithelium, *i.e.*, of the mammary gland (the lung tumor rates had not yet been demonstrated), while the tar painting test was applied to a different tissue—epithelium of the back. It was obvious that it would be a more interesting experiment to attempt to induce tumors in a tissue in respect to which the stock of mice already differed spontaneously. Since then the differences in lung tumor rates have been demonstrated, and the discovery by Murphy and Sturm (4) of a method of inducing tumors in the lung by tar painting presented the possibility of making the desired test.

Experiment 1.—30 agouti mice, 18 males and 12 females, between 2 and 3 months old, were selected from Strain 1194, and an equal number, 19 males and 11 females, from the Bagg albinos. The tar used was similar to that formerly employed, a residue obtained after distillation at 300°C., from which acids, bases, and phenols had been removed, and which was then dissolved in benzene. The novelty of the method lies in the loci which are subjected to treatment. The applications, instead of being made always on one spot, are distributed to 12 different regions of the body—on the dorsal and ventral surfaces, axilla, groin, neck, etc. The painting is done three times weekly, and four applications are made on each area, so that the treatment lasts approximately 4 months. After an interval of 6 more months, or when the animals are 12 to 13 months old, they are killed.

After such treatment very few mice develop skin tumors. Apparently no single region in the skin is irritated sufficiently to undergo neoplastic changes, but for some reason, as yet undetermined, a certain percentage of individuals will present primary tumors in the

lung. Since mice are subject to "spontaneous" tumors in the lung, it is necessary to differentiate between the spontaneous and tar-

Agouti mice from strain with low incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Jan.	Feb.	Mar.	Apr.	May	June	Skin	Lung - gross	Lung-microscopic
1	♀						†	—	—	—
2	♂						†	—	—	—
3	♂			†				—	—	—
4	♂						†	—	1 nod. in 1 lobe	+
5	♂		†					—	—	—
6	♀						†	—	—	—
7	♀		†					—	—	—
8	♀						†	—	Min. nod. in 1 lobe	+
9	♀			†				—	—	—
10	♀						†	—	Min. nod. in 1 lobe	—
11	♂		†					—	—	—
12	♂						†	—	—	—
13	♂		†					—	—	—
14	♂	†						—	—	—
15	♂						†	—	Min. nod. in 1 lobe	+
16	♀					†		—	—	—
17	♀	†						—	—	—
18	♂						†	Epithelioma	—	—
19	♀						†	—	Min. nod. in 1 lobe	—
20	♂	†						—	—	—
21	♂						†	Epithelioma	Min. nod. in 1 lobe	+
22	♂						†	—	2 • • 2 •	+?
23	♂			†				—	—	—
24	♂						†	—	—	—
25	♂						†	—	—	—
26	♂					†		—	—	—
27	♀			†				—	—	—
28	♀	†						—	—	—
29	♀		†					—	—	—
30	♂						†	—	Min. nod. in 1 lobe	+

Agouti mice surviving 6 months after tarring { 5 positive, 10 negative, 1 doubtful }

TEXT-FIG. 1.

induced growths. This is done by checking the age of the animals. As a rule, lung tumors do not develop spontaneously until later in

life. In our stocks 8 months is the earliest age at which such growths have been noted, and then in only 1 to 3 per cent of the individuals.

Albino mice from strain with high incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Jan.	Feb.	Mar.	Apr.	May	June	Skin	Lung-gross	Lung-microscopic
31	♂						†	—	Many nods in 4 lobes	+
32	♂						†	—	" " 5 "	+
33	♂						†	—	" " 4 "	+
34	♂						†	—	" " 5 "	+
35	♂						†	Hyperplasia	" " 3 "	+
36	♂				†			—	2 " 2 "	−
37	♂					†		—	—	−
38	♂				†			—	—	−
39	♂			†				Epithelioma	—	−
40	♂						†	—	Many nods in 3 lobes	+
41	♂						†	—	" " 4 "	+
42	♂						†	—	" " 4 "	+
43	♂						†	—	" " 5 "	+
44	♂						†	—	2 " 2 "	+
45	♂			†				—	Many " 3 "	+
46	♂			†				—	2 " 2 "	−
47	♂			†				—	Many " 2 "	−
48	♂			†				—	—	−
49	♂						†	—	Many nods in 4 lobes	+
50	♀						†	—	" " 4 "	+
51	♀						†	—	2 " 2 "	+
52	♀						†	—	Many " 5 "	+
53	♀						†	—	" " 5 "	+
54	♀						†	—	" " 4 "	+
55	♀						†	—	" " 4 "	+
56	♀						†	—	" " 5 "	+
57	♀						†	—	" " 4 "	+
58	♀						†	—	" " 4 "	+
59	♀						†	—	" " 3 "	+
60	♀						†	—	" " 4 "	+

Albino mice surviving 6 months after tarring { 22 positive, 0 negative, 0 doubtful }

TEXT-FIG. 2.

The youngest mouse in the Bagg strain in which a lung tumor has been found was 15 months old, and in the agoutis, 18 months. A

marked lowering of the tumor age or increase in tumor rate among mice which have been tarred in the way described may fairly be considered a result of the treatment.

Text-figs. 1 and 2 give the results of the experiment. Almost half of the agoutis died before the expiration of the 6 month interval. In the skin of 2 of the mice neoplastic changes had occurred; both animals proved to have carcinomas. At autopsy minute lesions or nodules were found in the lungs of 8 individuals, usually in only one lobe of the lung. Microscopic examination, however, revealed that only 5 mice had lung tumors, 1 was doubtful, and 10 were negative.

Of the albinos, a larger number lived to the end of the experiment. 1 that died the 4th month after tarring was stopped had an epithelioma on the back. 1 that died in the 3rd month after tarring had a primary lung tumor. All of the 22 mice that were killed after 6 months presented primary tumors of the lung, making a total of 23 positive and none negative. The large majority of them were multiple.

In this experiment the two strains gave different percentages of lung tumors after the tar treatment. Strain 1194, which "spontaneously" produces 6.73 per cent of lung tumors, produced 37.5 per cent when painted with tar. The rate of spontaneous lung tumors in the Bagg strain is 37.04 per cent, but when painted the incidence increased to 100 per cent. In both strains the rate increased, but the rate of the agouti stock did not increase sufficiently to overtake that of the albinos. A difference still exists between them.

The numbers of mice employed were not large, and it was, of course, desirable to repeat the experiment. The results, however, so strongly supported the conception of an hereditary difference in constitution in the animals of the two families that an experiment to test the point was carried out. If an hereditary difference persists even after tarring, then it should be possible to cross the two stocks and with the first filial generation thus obtained make backcrosses to the two original strains and demonstrate differences in the individuals of the backcross generation. In anticipation of the result actually obtained, mice in the first experiment had been crossed before the outcome of the experiment was known, and in the succeeding generation backcrosses were made after the discontinuance of tarring. For each generation a group from each of the original stocks was run at the

same time as controls. The controls are of the same nature as the original experiment and will be summarized after they have been described in detail.

F_1 from high tumor strain (Bagg albino) X low tumor strain (No. 1194)

Number	Sex	Length of life after tarring: Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Lesions: Skin	Lung — gross	Lung-microscopic
201	♂	—	—	—	—	—	—		1 lesion in 1 lobe	+
202	"	—	—	—	—	—	—		1 " " 1 "	+
203	"	—	—	—	—	—	—		2 " " 1 "	+
204	"	—	—	—	—	—	—		2 " " 2 "	+
205	"	—	—	—	—	—	—		3 " " 3 "	+
206	"	Died during tarring							0 " " 0 "	
207	"	—	—	—	—	—	—		3 " " 3 "	+
208	"	—	—	—	—	—	—		1 " " 1 "	−
209	"									
210	♀									
211	"						Living			
212	"									
213	"									
214	♂	—	—	—	—	—	—		8 " " 3 "	+
215	"	—	—	—	—	—	—		3 " " 3 "	+
216	"	—	—	—	—	—	—		0 " " 0 "	−
217	♀	—	—	—	—	—	—		4 " " 3 "	+
218	"	—	—	—	—	—	—		4 " " 3 "	+
219	"	—	—	—	—	—	—		3 " " 2 "	+
220	"	—	—	—	—	—	—		1 " " 1 "	+
221	♂	—	—	—	—	—	—		0 " " 0 "	−
222	"	—	—	—	—	—	—		1 " " 1 "	+
223	"	—	—	—	—	—	—		3 " " 2 "	+?
224	"	—	—	—	—	—	—		2 " " 2 "	+
225	"	—	—	—	—	—	—		0 " " 0 "	−
226	"	—	—	—	—	—	—		2 " " 2 "	+
227	"	—	—	—	—	—	—		2 " " 2 "	+
228	"	—	—	—	—	—	—		2 " " 2 "	+
229	"	—	—	—	—	—	—		5 " " 3 "	+
230	♀	—	—	—	—	—	—		5 " " 3 "	+
231	♂	—	—	—	—	—	—		2 " " 2 "	+
232	"	—	—	—	—	—	—		2 " " 2 "	+
233	♀	—	—	—	—	—	—		1 " " 1 "	?
234	"	—	—	—	—				0 " " 0 "	0
235	"	—	—	—	—	—	—		3 " " 2 "	+

22 positive = 79%
4 negative = 14%
2 doubtful = 7%

TEXT-FIG. 3.

Experiment 2.—Before the conclusion of the tar painting in Experiment 1, crosses between the two strains were made in which all of the females were used and all of the males with the exception of 2 agouti males, Nos. 21 and 24, and 7 albino males, Nos. 43 to 49 inclusive. Many of the crosses, however, were un-

productive of offspring either through the sterility of the parents or through destruction of the young. 13 of the matings were successful. In 6 instances the male parent was from the albino or high tumor strain, and in 7 cases from the agouti or low tumor strain. A few of the next generation were too sickly to be used, but 35 of the F_1 generation were selected to be tested by the same method of tarring as were their parents. The results are shown in Text-fig. 3. The painting was begun when the mice were 2 to 3 months old, as in the previous experiment.

Agouti mice from strain with low incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring					Lesions		
		Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Glands	Lung – gross	Lung-microscopic
251	♂	†						—	—	—
252	♀						†	—	Many nods in 4 lobes	—
253	♀						†	Mammary gland tumor	—	—
254	♀					†		—	—	—
255	♀						†	—	1 nod in 1 lobe	+
256	♂			†				—	No autopsy	
257	♂						†	—	1 nod in 1 lobe	—
258	♀						†	—	—	—
259	♂				†			—	—	—
260	♂					†		—	—	—
261	♀						†	—	—	—
262	♀						†	—	—	—
263	♂						†	—	—	—
264	♀						†	—	1 nod in 1 lobe	+
265	♀						†	—	1 " " 1 "	—
266	♂					†		—	—	—
267	♂						†	—	—	—
268	♀						†	—	—	—
269	♂	†						—	No autopsy	
270	♀						†	—	—	—

2 positive
11 negative
0 doubtful

TEXT-FIG. 4

The mortality was very low. 1 male, No. 206, died before the tarring was completed, and 1 female, No. 234, died 3 months after the treatment was over. Neither had a tumor of any kind. 1 male, No. 209, and 4 females, Nos. 210 to 213 inclusive, were not killed with the others. They came from parents both of which had lung tumors and were kept for breeding purposes. In two other matings the parents were positive and the three offspring (Nos. 229, 230, 231) also were positive. In two matings the parents were both negative but they had not lived

the allotted period. Of their offspring four (Nos. 202, 203, 204, 205) were positive and one (No. 206) died during treatment. The rest of the F_1 came from parents one of which had shown a lung tumor in the first experiment and the other had not. There were 28 in all of which 21 were males. At autopsy numerous lesions were discovered in the lung, and upon microscopical examination 22 mice were found to have primary tumors, 4 mice were negative, and 2 were doubtful; that is, 79 per cent had undoubted tumors. All of the negative animals were males.

As controls for this experiment, groups of mice from each of the two original families were submitted to the same experimental treatment. The first group

Albino mice from strain with high incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Aug	Sept	Oct	Nov	Dec	Jan	Skin	Lung - gross	Lung-microscopic
236	♂						†	—	Many nods in 5 lobes	+
237	♂						†	—	1	?
238	♀						†	—	3	+
239	♂						†	—	—	−
240	♂				†			—	—	−
241	♂						†	—	Many nods in 4 lobes	+
242	♂						†	—	1	+
243	♂			†				—	—	−
244	♀	†						—	Many nods in 3 lobes	−
245	♀	Died during tarring						—	—	−
246	♂						†	—	Many nods in 3 lobes	+
247	♂						†	—	3	+
248	♀						†	—	7	+
249	♂						†	—	2	+
250	♂						†	—	—	−

8 positive
2 negative
1 doubtful

TEXT-FIG. 5.

comprised 20 agouti mice from the low tumor Strain 1194, and the second group consisted of 15 mice taken from the Bagg albino stock. Again the age factor was checked, only mice of 2 to 3 months being used.

Text-figs. 4 and 5 present the results. 13 of the agoutis and 11 albinos lived to practically the end of the experiment. (A few are included which died 2 days to 2 weeks before the end.) A colored female, No. 253, had a mammary gland tumor. There were no lesions in the skin. Of the agoutis, 2 were positive and 11 negative, while among the albinos 8 were positive, 2 negative, and 1 doubtful.

Backcross from F_1 (Bagg albino x No. 1194) X Bagg albino (high tumor strain)

Number	Sex	Color	Length of life after tarring						Lesions			
			Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	Skin	Lung — gross		Lung-microscopic
340	♂	Agouti	—	—	—	—	—	—		∞ lesions in 4 lobes		+
341	"	Cin.	Died during tarring							0		
342	"	Albino	—	—	—	—	—	—		0	0	−
343	"	"	—	—	—	—	—	—		2	2	+
344	"	"	—	—	—	—	—	—		∞	5	+
345	"	"	—	—	—	—	—	—		4	4	+
346	"	"	—	—	—	—	—	—		∞	3	+
347	♀	"	—	—	—	—	—	—		4	4	?
348	"	"	—	—	—	—	—	—		∞	3	+?
349	"	"	—	—	—	—	—	—		3	3	+
350	"	"	—	—	—	—	—	—		∞	5	+
351	"	"	—	—	—	—	—	—		0	0	−
352	♂	"	—	—	—	—	—	—		3	2	+
353	"	Agouti	—	—	—	—	—	—		2	2	+
354	"	Albino	—	—	—	—	—	—		5	4	+
355	"	"	Died during tarring							0	0	
356	♀	Agouti	—	—	—	—	—	—		3	3	+
357	"	Albino	—	—	—	—	—	—		6	3	+
358	"	"	—	—	—	—	—	—		2	2	+
359	"	Agouti	—	—	—	—	—	—		4	2	+
360	"	"	Died during tarring									
361	"	"	—	—	—	—	—	—		4	3	+
362	"	Albino	—	—	—	—	—	—		0	0	−
363	"	"	—	—	—	—	—	—		6	3	+
364	"	"	—	—	—	—	—	—		3	3	+
365	"	"	—	†						0	0	−
366	♂	"	—	—	—	—	—	—		8	4	+
367	"	"	—	—	—	—	—	†		0	0	−
368	♀	Agouti	—	—	—	—	—	—		∞	4	+
369	"	"	—	—	—	—	—	—		∞	5	+
370	"	Albino	—	—	—	—	—	—		8	3	+
371	"	Black	—	—	—	—	—	—		4	3	+
372	"	Agouti	—	—	—	—	—	—		3	3	+
373	"	"	—	—	—	—	—	—		2	2	−?
374	"	Albino	—	—	—	—	—	—		5	3	+
375	♂	Agouti	—	—	—	—	—	—		3	2	+
376	"	Black	—	—	—	—	—	—		6	4	+
377	"	Albino	—	—	—	—	—	—		∞	4	+
378	"	"	—	—	—	—	—	—		∞	3	+
379	♀	Agouti	—	—	—	—	—	—		∞	5	+
380	"	Albino	—	—	—	—	—	—		1	1	−
381	"	"	—	—	—	—	—	—		2	2	+

	♂♂		♀♀		
	Pos.	Neg.	Pos.	Neg.	Doubtful
Colored	4	0	8	0	1
Albino	9	1	9	3	2
Total	13	1	17	3	3

30 positive = 81%
4 negative = 11%
3 doubtful = 8%

TEXT-FIG. 6.

Backcross from F_1 (Bagg albino x No.1194) X No.1194 (low tumor strain)

Number	Sex	Length of life after tarring						Lesions			
		Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	Skin	Lung — gross		Lung-microscopic
301	♂						†		3 lesions in 2 lobes		+
302	"						†		0 " " 0 "		−
303	"						†		2 " " 1 "		+
304	♀						†		5 " " 2 "		+
305	"						†		0 " " 0 "		−
306	"						†		5 " " 2 "		+
307	"						†		0 " " 0 "		−
308	"						†		1 " " 1 "		−
309	♂						†		0 " " 0 "		−
310	"						†		∞ " " 3 "		+
311	"						†		2 " " 2 "		−
312	♀						†		2 " " 2 "		−
313	"						†		1 " " 1 "		+
314	"						†		1 " " 1 "		−
315	"						†		3 " " 3 "		−
316	"						†		1 " " 1 "		−
317	"						†		2 " " 2 "		−
318	"						†		0 " " 0 "		−
319	"						†		0 " " 0 "		−
320	"						†		2 " " 2 "		+
321	"						†		2 " " 2 "		−
322	♂						†		0 " " 0 "		−
323	"						†		1 " " 1 "		−
324	"			†					0 " " 0 "		−
325	"						†		1 " " 1 "		−
326	"						†		2 " " 2 "		+
327	"						†		1 " " 1 "		+
328	"						†		1 " " 1 "		+
329	♀						†		1 " " 1 "		+
330	"						†		3 " " 2 "		−
331	"						†		1 " " 1 "		+
332	♂						†		0 " " 0 "		−
333	"						†		2 " " 2 "		+
334	"						†		1 " " 1 "		−
335	"						†		0 " " 0 "		−
336	"						†		∞ " " 2 "		+
337	"						†		0 " " 0 "		−
338	"						†		1 " " 1 "		−
339	"						†		1 " " 1 "		+

♂		♀	
Pos.	Neg.	Pos.	Neg.
9	11	6	12

15 positive = 39%
23 negative = 61%

TEXT-FIG. 7.

In this experiment we may note that the cross between the two stocks gives a high percentage of tumors. Among the controls painted at the same time, the individuals from the high tumor strain showed a greater number of pulmonary tumors than did those from the low tumor strain—just as they had in the first experiment. Among the affected mice, the number of nodules and the number of lobes involved were greater in those from the high tumor stock than in the low.

Agouti mice from strain with low incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring: Nov. Dec. Jan. Feb. Mar. Apr.	Lesions: Skin	Lung – gross	Lung-microscopic
382	♂	†	—	—	–
383	♀	†	—	1 nod. in 1 lobe	–
384	♀	†	—	2 • • 2 •	+
385	♀	†	—	2 • • 2 •	+
386	♂	†	—	1 • • 1 •	–
387	♂	†	—	1 • • 1 •	–
388	♀	†	—	1 • • 1 •	–
389	♀	†	—	—	–
390	♀	†	—	—	–
391	♂	†	—	2 nods. in 2 lobes	–
392	♂	†	—	—	–
393	♀	†	—	1 nod. in 1 lobe	–
394	♂	†	Hyperplasia	1 • • 1 •	–
395	♂	†	—	—	–
396	♂	†	—	1 nod. in 1 lobe	+
397	♀	†	—	—	–
398	♂	†	—	—	–
399	♀	†	—	1 nod. in 1 lobe	–
400	♀	†	—	—	–
401	♀	†	—	—	–

3 positive
11 negative
0 doubtful

TEXT-FIG. 8.

Experiment 3.—Four groups of mice at the age of 2 to 3 months were tarred as in the preceding experiment. Two series were mice obtained from backcrosses between the first filial generation and the two original strains, and two series were mice from the original strains and were used as controls.

Group 1. Backcross from the High Tumor Strain.—These mice were obtained by crossing sons belonging to the first filial generation with the Bagg albinos

or high tumor strain. Each male was mated with several females. In a few cases one of the females used was the maternal parent from Experiment 1, but no young suitable for the experiment were obtained from them. The mothers of the offspring used were from stock. They had not been treated by tar painting, so that it is not known how many or which individuals would have been susceptible to tar-induced growths in the lung. 42 offspring were obtained, and the results of tarring are shown in Text-fig. 6. 3 mice died during tarring, and 1 in the 1st and 1 in the 5th month after tarring. At autopsy it was

Albino mice from strain with high incidence of spontaneous lung tumors

Number	Sex	Length of life after tarring						Lesions		
		Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	Skin	Lung-gross	Lung-microscopic
402	♂			†				—	—	−
403	♂	†						—	—	−
404	♂						†	—	Many nods. in 5 lobes	+
405	♀						†	—	· · ·5 ·	+
406	♀	Died during tarring						—	No autopsy	
407	♂						†	—	Many nods. in 4 lobes	+
408	♂						†	—	· · ·2 ·	+
409	♂		†					—	1 · ·1 ·	+
410	♂						†	—	Many · ·3 ·	+
411	♂						†	—	· · ·2 ·	+
412	♂						†	—	1 · ·1 ·	−
413	♂						†	—	1 · ·3 ·	+
414	♂					†		—	—	−
415	♂			†				—	—	−
416	♂						†	—	2 nods. in 2 lobes	+
417	♂						†	—	3 · ·3 ·	+
418	♂						†	—	3 · ·3 ·	−
419	♂						†	—	—	−
420	♂						†	—	2 nods. in 2 lobes	−

10 positive
4 negative
0 doubtful

TEXT-FIG. 9.

found that the lesions in the affected individuals were very numerous. Among 14 males, all but 1 were positive, while among 23 females, 17 showed tumors of the lung, 3 were without them, and 3 were doubtful. If the two groups are taken together and the 3 doubtful cases classed as negative, the ratio is 30 with tumors and 7 without, or 81.1 per cent tumor mice. This rate is about the same as that of the F_1 and of the albino stock.

Group 2. Backcross from the Low Tumor Strain.—The same F_1 males which were backcrossed to the albino stock were also mated with females from Strain 1194, the low tumor strain. 39 offspring resulted and all were agouti as expected. 1 died the 3rd month after tarring. The ratio (Text-fig. 7) for the males was 9 with tumors to 11 without tumor, for the females, 6 positive to 12 negative. The two classes taken together give 15 positive to 23 negative, or 39.47 per cent. This is only about half the number of tumors found in the F_1 or the backcross group from the high tumor strain.

Group 3. Control from Low Tumor Strain.—20 control mice (9 males and 11 females) were selected from Strain 1194, Text-fig. 8. None died during the treatment. When killed, 1 showed an epithelial proliferation on the back of the neck at one of the painted areas, but it had not progressed far enough to be called an epithelioma. Only 3 of the animals produced tumors in the lung; the other 17 were negative.

Group 4. Control from the High Tumor Strain.—The control from the albino group consisted of 19 mice, of which 17 were males, Text-fig. 9. 6 died before the

	Strain 1194			Bagg albino		
	Tumor	Non-tumor	Doubtful	Tumor	Non-tumor	Doubtful
Exp. 1	5	10	1	23	0	0
" 2	2	11	0	8	2	1
" 3	3	17	0	10	4	0
Total	10	38	1	41	6	1

Strain 1194 11 tumor : 38 non-tumor = 22.45% ± 4.02
Albino 41 " : 7 " " = 85.42% ± 3.43
Difference = 62.97% ± 5.29

TEXT-FIG. 10.

end of the experimental period. The lesions in the lung were more numerous than in the preceding group, both as to number of individuals and number of lobes affected. Tumors were found in 9 of the 13 survivors. 1 of the mice dying only 2 months after the discontinuance of the tar treatment had a lung tumor, so that the ratio is 10 to 4. In this case also the albino mice from the high tumor strain give a greater percentage of tar tumors than do the agoutis from the low tumor strain.

In the first experiment samples of mice from two strains which differ in their spontaneous tumor rates were contrasted when subjected to a method of tarring which produces lung tumors. The control groups in Experiments 2 and 3 were repetitions of the same test, so that the three experiments may be compared from that point

of view. In each of the three trials, the colored (low tumor strain) and the albino (high tumor strain) groups differed from each other in their reaction, Text-fig. 10. During the series of experiments the percentage of tumors decreased in the case of the agouti strain but increased in the albinos; however, the percentage was always lower in the agoutis and higher among the albinos. The direction of the difference between the two was constant. Combining the figures for all of the trials gives a ratio of 11 tumors to 38 non-tumors for Strain 1194, or 22.45 per cent $\pm$ 4.02. For the Bagg albinos the ratio is 41 tumors to 7 non-tumors, or 85.42 per cent tumors $\pm$ 3.43. The difference between the per cents is 62.97 $\pm$ 5.29. Since the difference is more than eleven times its probable error, it is undoubtedly significant.

Since a distinct difference in the two families is, therefore, shown, it is interesting to note the results of their crossing. Experiment 2 (Text-fig. 3) shows that the F_1 generation when tarred, as were the parents, gives 79 per cent of tumors. This is almost as high a rate as that given by the more susceptible parent strain—the Bagg albinos, which gave 85 per cent of lung tumors. When the F_1 were backcrossed to the high tumor strain, a high rate (81 per cent) was again shown, but when backcrossed to the low tumor strain, Strain 1194, the rate dropped about half, 39 per cent. It seems evident that the percentage of tumors to be obtained in the lungs of mice after tarring depends upon the strain of mice employed and may be controlled by backcrossing the hybrids to one or the other of the strains used.

The data are insufficient to make possible an accurate determination of the number of hereditary factors involved. From the high tumor rate given by the first filial generation it would seem that at least one factor for susceptibility is dominant or partially so. On the basis of the "dominant hypothesis," however, we might expect a higher percentage than 39 in the backcross. Further generations will settle the question.

In solving the problem of the genes involved, help may possibly be obtained from a more detailed study of the histology of the tumors. The tumors do not all present the same picture. The majority are formed of cuboidal cells, somewhat resembling the epithelium of the terminal bronchi. Others are composed of columnar cells, similar to the cells of bronchial epithelium, which, supported by

strands of connective tissue, thrust out finger-like processes. They are comparable to the papillary cyst-adenomas described by Tyzzer. There are many gradations between the types. Some tumors have not the appearance of being malignant, while others show examples of mitosis and definite infiltration. In one case the cells were elongated almost to a spindle shape and were quite unlike either of the types already mentioned. So far a satisfactory classification of the types which would permit identifying them as different genetic classes has not been worked out. This must remain a problem for future investigation.

Another point to be considered is the adequacy of this method of tarring as a test of susceptibility. A slightly different procedure; such as a longer period of tarring, more frequent applications, a longer period between tarring and autopsy, or even a different tar preparation, might yield a higher percentage of tumors. It is possible that the albino strain which gives positive results in 85 per cent of the cases may really be a 100 per cent strain genetically but not show it because of the ineffectiveness of the method employed. In that case the agoutis also might give a higher per cent than 22. Variations in procedure have not been tested. At present we can merely state the results obtained by the method described.

SUMMARY AND CONCLUSIONS.

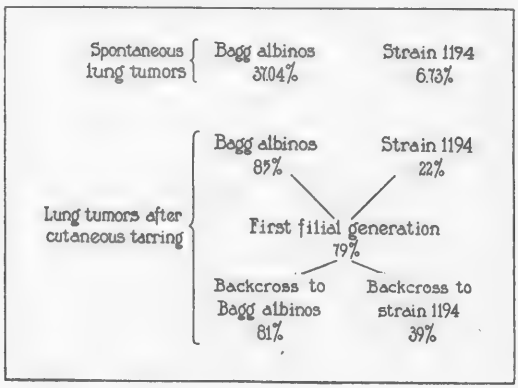
Evidence has previously been submitted in favor of the theory that susceptibility to spontaneous tumors of various types is inherited. The question arose whether susceptibility to tumors induced by tar could be shown to be hereditary by experimenting with the same strains of mice which had already been shown to differ significantly in respect to their spontaneous tumor rates.

Two strains of mice were selected for observation. One strain, the Bagg albinos, has a low rate of mammary gland tumors but a higher rate of spontaneous lung tumors. The other strain, No. 1194, agouti, has a higher rate of mammary gland tumors but a lower rate of tumors of the lung. A previous test showed no difference in the percentages of skin tumors which arose after tar painting.

It has already been shown that the difference in the lung tumor rates

is mathematically significant. When the two stocks are treated with tar by applying the irritant, not in the same spot, but on different areas successively, the percentage of lung tumors is increased in each stock, Text-fig. 11. The rate of the Bagg albinos increased from 37.04 to 85 per cent, that of Strain 1194 from 6.73 to 22 per cent. But a difference between them is still maintained, and this difference also is significant mathematically.

When the two strains are crossed and the offspring subjected to the tar treatment, the latter give a high percentage of lung tumors—79 per cent in 28 individuals—about the same as the parental high tumor strain. When the F_1 sons are backcrossed to the original



TEXT-FIG. 11.

stocks, the cross to the high tumor strain maintains the high tumor rate, 81 per cent in 37 mice, while in the cross to the low tumor strain the percentage drops to 39 per cent in 38 mice. This result indicates that susceptibility to tar-induced tumors in the lung is hereditary. The number of factors concerned has not yet been ascertained. Possibly one or more of them is dominant.

In general, the conception that susceptibility to pulmonary tumors is hereditary seems to be upheld by the fact that the two strains of mice described differ conspicuously in respect to spontaneous tumor rates under ordinary laboratory conditions; the strains differ also

under experimental conditions, as described in this report; and when crossed, their offspring by suitable backcrosses, will again show significant differences.

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FURTHER STUDIES CONCERNING THE FILTRABLE VIRUS PRESENT IN THE SUBMAXILLARY GLANDS OF GUINEA PIGS.

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In a previous paper (1), the occurrence of a filtrable virus in the submaxillary glands of full grown guinea pigs was reported. Emulsions of the submaxillary glands containing potent virus were found to be active after filtration through a Berkefeld candle. The virus was thermolabile, resistant to 50 per cent glycerol, and could not be cultivated either in the presence or absence of air. The various criteria necessary to establish the presence of a filtrable virus were thus fulfilled. It was found extremely difficult, however, to transmit the virus continuously from animal to animal in series. In spite of the fact that young guinea pigs, inoculated intracerebrally,[1] died with great regularity, it was found impossible to transmit the virus from brain to brain. It was only by changing the site of inoculation, as for instance, inoculating first in the brain and then in the testicle, or *vice versa*, that it was possible to carry the virus through two animals in series. In only one instance was the virus transmitted through three young guinea pigs in series alternating as follows: testicle-brain-testicle. In subsequent work the virus has occasionally been transmitted from brain to brain by using very large doses or by making multiple injections. But the second animal in the series in no instance died or was severely sick. The reaction in the second guinea pig of a series, whether the site of inoculation was changed or remained the same, was always very much milder than in the first guinea pig.

In this paper the results of further attempts to transmit the guinea pig virus to other species of animals are reported, certain immuno-

[1] The animals were anesthetized with ether before inoculation.

logical aspects of this disease are discussed, and finally, the description of two methods of transmitting the virus from guinea pig to guinea pig in series is given.

Transmission to Other Species of Animal.

It was thought that if it were possible to infect another species of animal with the guinea pig virus the problem of the transmission of the active agent from animal to animal would become less difficult. It has previously been reported that the intracerebral inoculation of the guinea pig virus into young rabbits, rats, and kittens proved negative. The observations have now been extended to include chickens, pigeons, dogs, a monkey (*Macacus rhesus*), and mice. All these animals, however, proved refractory to intracerebral inoculation[1] of the virus. Direct inoculations of the guinea pig virus into the submaxillary glands of young rabbits and young rats were also made. The glands were removed at various periods following inoculation and examined histologically. No lesions containing nuclear inclusion bodies were found.

Active Immunity.

An important characteristic of filtrable viruses is that in most instances it is possible to produce an active immunity against the agents by the inoculation of small amounts of potent material. The injection of heat-killed virus on the other hand, in contrast with the results obtained with bacteria, fails to produce immunity.

Experimental.—A group of young guinea pigs were divided into two lots; each animal of one lot received three subcutaneous injections of active guinea pig virus on 3 successive days, the guinea pigs of the other lot remained untreated. 2 weeks after the last subcutaneous inoculation, the guinea pigs from both lots were inoculated intracerebrally with active guinea pig virus. The guinea pigs which had received the subcutaneous injections survived, and, with a few exceptions, those which had remained untreated died in the usual period of time. In all the guinea pigs which received the subcutaneous injections of the guinea pig virus and which later proved refractory to brain inoculation, the specific lesion of the virus was found to be present in the submaxillary glands. It was thought, therefore, that the immunity to intracerebral inoculation depended on an active infection of the submaxillary glands and, furthermore, that the virus travelled from the skin to the gland. In order to test this hypothesis, further experiments were undertaken.

It was necessary to determine whether the specific lesion could regularly be found in the submaxillary gland following subcutaneous injections of active guinea pig virus, and if so, how soon after inoculation the lesion was demonstrable. Since infection of the submaxillary gland occurs spontaneously in uninoculated guinea pigs, it was necessary to observe a control group of guinea pigs of approximately the same age over a similar or a longer period of time.

Methods.—The virus used for injection was prepared as follows. The submaxillary glands of two or more adult guinea pigs were removed with sterile precautions. A small piece of each gland was cut off and prepared for histological examination. In experiments in which it was necessary to make injections of virus on 2 or 3 successive days, another piece of each gland was put in 50 per cent glycerol and kept in the ice box until used. The rest of the material was ground thoroughly with sand in a mortar and the emulsion suspended in Locke's solution. Approximately, four glands were suspended in 2 cc. of fluid. The emulsion was centrifuged at low speed for a few moments, and the supernatant fluid used for injection. Aerobic and anaerobic cultures were made from the fluid to make sure that no bacteria were present.

Sections prepared from the submaxillary glands used for injection were always examined to determine whether acidophilic nuclear inclusion bodies were present in the duct cells, and if so, their number and size were noted. Furthermore, 0.1 cc. of the supernatant fluid was inoculated intracerebrally into young guinea pigs to determine the virulence of the particular emulsion. These animals usually died 5 or 6 days following injection and sections were prepared from their brains. In the majority of instances, meningitis was present and the exudate contained cells with acidophilic nuclear inclusion bodies.

Every effort was made to use for inoculation only guinea pigs less than 1 month old. It is, however, extremely difficult to judge the age of guinea pigs by their size. In certain experiments in which it was particularly important to exclude the possibility of a spontaneous infection of the submaxillary glands, only guinea pigs of known age were used. In other experiments where the age of guinea pigs used was uncertain, one submaxillary gland was removed from each guinea pig at the beginning of the experiment[2] and examined histologically to make certain that the animal was not already infected.

Series E, September 16, 1926.—Seven young guinea pigs, approximately 1 week of age, were selected, and the left submaxillary gland removed from all of them and prepared for histological examination. These guinea pigs were then divided into two groups, three—Nos. 69-7, 69-8, 70-2—were kept together in one cage and left untreated, and four—Nos. 70-3, 70-4, 70-5, 70-7—received three subcutaneous injections on 3 successive days of 0.1 cc. of an emulsion of active guinea pig virus. No definite local reaction occurred at the site of inoculation.

The specific lesion was not found in the left submaxillary glands removed from each guinea pig in both groups at the beginning of the experiment. 6 days after the first subcutaneous inoculation, the right submaxillary gland was removed from Guinea Pig 70-7. Microscopic study of this gland failed to show the specific

[2] All operations were performed under anesthesia.

lesion. 12 days after the first inoculation, the right submaxillary gland was removed from Guinea Pig 70-4. Microscopic study of this gland showed the presence of typical nuclear inclusion bodies in the duct cells. The inclusion bodies were of the small type, indicating an early lesion. 18 days after the first inoculation the right submaxillary glands were removed from Guinea Pigs 70-3 and 70-5. On histological examination these glands showed typical nuclear inclusion bodies both of the small and large type in the duct cells. 18 days after the beginning of the experiment the right submaxillary glands of the three control guinea pigs were removed. On microscopic study the specific lesion was not found in any of these glands.

This experiment seemed to indicate that young guinea pigs which are injected subcutaneously with active guinea pig virus become infected more rapidly than untreated guinea pigs of approximately the same age and, furthermore, that it requires 12 to 18 days for the specific lesion in the duct cells of the glands to develop. No macroscopic lesion, beyond a little induration, developed at the site of the subcutaneous inoculations.

Further experiments, Series F, K, and R, were made to confirm these observations and to determine whether guinea pigs, which had been injected subcutaneously with virus, subsequently became refractory to intracerebral inoculation. The results of intravenous and intraperitoneal injections of virus were also studied. As controls, a number of guinea pigs were injected subcutaneously with heat-killed virus, and the susceptibility of these animals to intracerebral inoculation was tested. In each experiment, several guinea pigs were not treated, and the submaxillary glands of these animals were removed at the same time that the glands were removed from the inoculated animals.

Table I shows the results of subcutaneous, intraperitoneal, and intravenous injection of guinea pig virus. It will be seen that one guinea pig in the control group, No. 73-2, was already infected at the beginning of the experiment, but that none of the other guinea pigs in this group developed the specific lesion during the time they were under observation.

In none of the guinea pigs of the other groups were lesions present at the beginning of the experiment. 13 to 14 days after inoculation, however, acidophilic inclusion bodies were present in the right submaxillary glands of all these guinea pigs with the exception of one, No. 74-2. In this animal, however, it was doubtful whether the whole dose was injected into the vein.

Many of the guinea pigs in this experiment were greatly weakened by the two operations for the removal of the submaxillary glands, and died of intercurrent infections. Three guinea pigs, Nos. 73-1, 73-3, and 73-5, in the control group, and three guinea pigs, Nos. 71-9, 72-1, and 72-7, which survived the two operations, were inoculated intracerebrally with 0.1 cc. of active guinea pig virus. Guinea Pigs 73-1, 73-3, and 73-5, developed meningitic symptoms and were moribund on the 8th day. Sections prepared from the brains of these animals showed a typical mononuclear, meningeal exudate with cells containing acidophilic nuclear inclusion bodies. Guinea Pigs 71-9, 72-1, and 72-7 remained well.

TABLE I.

Series F.

G. P.	Removal of gland at beginning of experiment						Removal of other gland				
	Gland	Date	Section	Result	Inoculum: virus	Site	Gland	Date	Day	Section	Result
73-1	Left	Oct. 2	646b	Neg.	0, controls		Right	Oct. 15	13th	694b	Neg.
73-2	"	"	647b	**Pos.**			"	"	"	695b	**Pos.**
73-3	"	"	648b	Neg.			"	"	"	696b	Neg.
73-4	"	"	649b	"			"	"	"	697b	"
73-5	"	"	650b	"			"	"	"	698b	"
73-6	"	"	651b	"			"	"	"	699b	"
73-8	"	"	653b	"			"	"	"	700b	"
74-0	"	"	655b	"			"	"	"	701b	"
71-8	"	Oct. 1	633b	"	Oct. 1,2 0.1 cc.	Subc.	"	"	14th	687b	**Pos.**
71-9	"	"	639b	"	" "	"	"	"	"	688b	**Pos.**
72-1	"	"	636b	"	" "	"	"	"	"	689b	**Pos.**
72-7	"	"	642b	"	" "	"	"	"	"	692b	**Pos.**
72-2	"	"	637b	"	Oct. 1 "	I.P.	"	"	"	690b	**Pos.**
72-6	"	"	641b	"	" "	"	"	"	"	691b	**Pos.**
72-8	"	"	643b	"	" "	"	"	"	"	693b	**Pos.**
74-1	"	Oct. 2	656b	"	" "	I.V.	"	"	13th	702b	**Pos.**
74-2	"	"	657b	"	" "	"?	"	"	"	703b	Neg.
74-3	"	"	658b	"	" "	"	"	"	"	704b	**Pos.**
74-4	"	"	659b	"	" "	"	"	"	"	705b	**Pos.**

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of submaxillary glands.
Negative indicates that no lesions were found in the glands.

TABLE II.

Series K.

G. P.	Removal of gland at beginning of experiment							Removal of the other gland				
	Gland	Date	Section	Result	Inoculum: virus			Gland	Date	Day	Section	Result
90-4	Left	Nov. 18	803b	Neg.	0, controls			Right	Dec. 3	15th	894b	Neg.
90-5	"	"	804b	"				"	"	"	895b	"
90-6	"	"	805b	"				"	"	"	896b	"
90-7	"	"	806b	"				"	"	"	897b	"
90-8	"	"	807b	"				"	"	"	898b	"
90-9	"	"	808b	"				"	"	"	899b	"
91-0	"	"	809b	"				"	"	"	900b	"
91-1	"	"	810b	"				"	"	"	901b	"
91-2	"	"	811b	"				"	Nov. 29	11th	862b	"
91-3	"	"	812b	"				"	Dec. 3	15th	902b	"
91-4	"	"	813b	"				"	"	"	903b	"
91-5	"	"	814b	Pos.				"	"	"	904b	Pos.
					Date	Amount	Site					
91-6	"	"	815b	Neg.	Nov. 18, 19	0.1 cc.	Subc.	"	"	"	905b	Pos.
91-7	"	"	816b	"	"	"	"	"	"	"	906b	Pos.
91-8	"	"	817b	"	"	"	"	"	"	"	907b	Pos.
91-9	"	"	818b	"	"	"	"	"	Nov. 27	9th	859b	Pos.
92-0	"	"	819b	"	"	"	"	"	Dec. 2	14th	883b	Pos.
92-1	"	"	820b	"	"	"	"	"	Dec. 3	15th	908b	Pos.
92-2	"	"	821b	"	"	"	"	"	"	"	909b	Pos.
92-3	"	"	822b	"	"	"	"	"	"	"	910b	Pos.
92-4	"	"	823b	"	"	"	"	"	Nov. 27	9th	860b	Neg.
92-5	"	"	824b	"	"	"	"	"	"	"	861b	Pos.
92-6	"	"	825b	"	"	"	"	"	Dec. 3	15th	911b	Pos.
92-7	"	"	826b	"	"	"	"	"	"	"	912b	Pos.

					Heat-killed							
93-1	"	Nov. 19	830b	"	Nov. 19, 20	0.1 cc.	Subc.	"	"	14th	913b	Neg.
93-3	"	"	832b	"	"	"	"	"	"	"	914b	"
93-4	"	"	833b	"	"	"	"	"	"	"	915b	"
93-5	"	"	834b	"	"	"	"	"	"	"	916b	"
93-6	"	"	835b	"	"	"	"	"	"	"	917b	"
93-7	"	"	836b	"	"	"	"	"	"	"	918b	"
93-8	"	"	837b	"	"	"	"	"	"	"	919b	"

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.
Negative indicates that no lesions were found in the glands.

Table II shows the results of subcutaneous injections of active and heat-killed guinea pig virus. It has previously been shown (1) that the guinea pig virus is inactivated by exposure to a temperature of 56°C. for a period of 1 hour.

It will be seen from Table II that whereas all except one of the animals injected with active virus developed lesions in the submaxillary glands, none of the guinea pigs injected with heat-killed virus showed acidophilic nuclear inclusion bodies in the duct cells of the glands.

On repetition of this experiment (Series R), similar results were obtained. None of the guinea pigs injected with heat-killed virus developed lesions of the submaxillary glands, in contrast to those animals which had been injected with active virus. The guinea pigs treated with heat-killed material succumbed to intracerebral inoculation of active virus, whereas the animals which had been injected subcutaneously with potent material, survived the same intracerebral injection. Sections prepared from the brains of the animals that died showed a typical meningeal exudate containing cells with acidophilic nuclear inclusion bodies.

Summary of Experiments. Series F, K, R.—The submaxillary glands of twenty-seven young guinea pigs which served as controls in these experiments were examined for the specific lesion. Three of these guinea pigs were found to be infected with the virus at the beginning of the experiment. None of the other twenty-four guinea pigs developed the lesion during the 2 weeks that they were under observation. Although it is impossible to be certain that a submaxillary gland is absolutely negative without cutting serial sections, further evidence that these guinea pigs were actually not infected is brought by the fact that they remained susceptible to intracerebral inoculation of the guinea pig virus. With the exception of the three guinea pigs mentioned above, which were spontaneously infected, all the guinea pigs in this group became sick following the injection of guinea pig virus into the brain. Histological sections of the brains of twelve of the twenty-four guinea pigs showed a typical meningeal exudate.

The submaxillary glands of twenty-two guinea pigs which received either one or two subcutaneous inoculations of active virus, were examined for the presence of acidophilic nuclear inclusions in the duct cells at intervals varying from 9 to 15 days following injection. With the exception of three cases, in one of which the animal died early (9th day), the submaxillary glands of these guinea pigs showed the specific lesion. The immunity of seventeen of these guinea pigs

was tested by intracerebral inoculation, twelve survived, three died acutely 1 or 2 days after inoculation, and two died late from intercurrent infections.

The submaxillary glands of three guinea pigs which had received one intraperitoneal inoculation of active guinea pig virus, were examined 14 days following injection, and all showed acidophilic nuclear inclusion bodies in the duct cells.

The submaxillary gland of four guinea pigs which had received one inoculation of active guinea pig virus intravenously, were examined 13 days following injection. Acidophilic nuclear inclusion bodies were found in three of them. The submaxillary gland in one case was found to be negative, but it is questionable whether, in this instance, the whole dose was injected into the vein.

The submaxillary glands of fourteen guinea pigs which received one or two subcutaneous inoculations of heat-killed virus were examined 14 days after inoculation. No acidophilic nuclear inclusion bodies were found in any of them. The immunity to intracerebral inoculation was tested in seven of these guinea pigs. They all developed meningitic symptoms. Sections prepared from the brains of three of these animals showed a typical meningeal exudate with acidophilic nuclear inclusion bodies.

It was thought of interest to determine whether young guinea pigs from which both submaxillary glands were removed and which were then injected subcutaneously with active guinea pig virus, would also become refractory to intracerebral inoculation.

Series L. December 1, 1926.—Fourteen young guinea pigs, all of approximately the same age, were selected and divided into two groups. Both submaxillary glands were removed from every guinea pig in Group I. The right submaxillary gland was removed from every guinea pig in Group II. On subsequent histological examination, it was found that none of the submaxillary glands removed at the beginning of the experiment showed the specific lesion. All the guinea pigs in Group I and in Group II received two subcutaneous injections on 2 successive days, December 2 and 3, of 0.1 cc. of active guinea pig virus.

12 to 15 days after the first subcutaneous injection, the left submaxillary gland was removed from every guinea pig in Group II. Microscopic study of these glands showed that in every instance the specific lesion had developed following the subcutaneous inoculation of the virus.

On December 17, 15 days after the first subcutaneous inoculation, all the

guinea pigs in Groups I and II which survived were inoculated intracerebrally with 0.1 cc. of active guinea pig virus. Furthermore, two guinea pigs, Nos. 85-6 and 85-8, which had had both submaxillary glands removed 6 weeks before, but which had received no injections, as well as one young normal guinea pig, No. 10–38, were also inoculated into the brain. These three animals, Nos. 85-6, 85-8, and 10-38, all showed typical symptoms and were moribund on the 6th day. Sections prepared from the brains of these animals showed typical lesions with cells containing acidophilic nuclear inclusion bodies. None of these guinea pigs in Groups I and II became sick.

The previous experiments had seemed to indicate that intracerebral immunity depended upon the localization of the virus in the submaxillary gland, but the result of this experiment showed that the presence of the glands was not essential for the development of immunity. An attempt was therefore made to determine whether in this case another localization of the virus had occurred.

In addition to the submaxillary glands, which lie fairly deep in guinea pigs, there is a superficial salivary gland which lies directly over the jugular vein and which probably corresponds to the parotid[3] in man. In the majority of full grown guinea pigs in which the submaxillary glands show the specific lesion no acidophilic nuclear inclusion bodies are present in this superficial gland and emulsions of this gland are not infectious when inoculated into the brains of young guinea pigs. The parotid glands of four guinea pigs of Group I were examined, and the specific lesion was found in three, and it seems, therefore, that, in the absence of the submaxillary glands, the virus localizes in the parotid glands, and the animals then become refractory to intracerebral injection. Theoretically, it would be of interest to inject guinea pigs from which both the parotid and submaxillary glands had been removed, to see where the virus would localize under these circumstances, but the position of the parotid glands is such that it is impossible to remove them completely without ligating both jugular veins.

On repetition of this experiment (Series Q) similar results were obtained. Seven guinea pigs from which both submaxillary glands had been removed, but which had received no injections of guinea pig

[3] To make the description more simple this gland has been called "parotid gland" in the discussion which follows.

virus, remained susceptible to brain inoculation. Four guinea pigs which had had both submaxillary glands removed and had received one injection of active guinea pig virus were refractory to intracerebral injection, and the specific lesion could be demonstrated in the parotid glands in three of these four animals.

The parotid glands of two guinea pigs which had one submaxillary gland left when they were injected subcutaneously with active guinea pig virus, showed acidophilic nuclear inclusion bodies, both in the submaxillary and the parotid glands. It seems, therefore, that following injections of active virus the parotid glands became infected even though one submaxillary gland was still present. When the infection occurs spontaneously, however, the submaxillary glands and not the parotid glands are usually the first to be infected. This point is brought out by the following experiment.

Series O. December 28, 1926.—The right submaxillary glands were removed from four young guinea pigs, Nos. 10-61, 10-63, 10-64, 10-65. These four animals were kept together in the same cage and left untreated. The left submaxillary glands were removed at various intervals and examined histologically. No acidophilic nuclear inclusion bodies were found in any of the sections prepared from the submaxillary glands removed at the beginning of the experiment. On February 1, 1927, approximately 1 month after the beginning of the experiment, the left submaxillary and parotid glands were removed from Guinea Pig 10-63. The specific lesion was found in the sections prepared from the submaxillary glands, but not in those prepared from the parotid gland.

On March 1, 1927, approximately 2 months after the beginning of the experiment, the left submaxillary and parotid glands were removed from Guinea Pig 10-64. The specific lesion was found in sections prepared from the submaxillary gland, but not in those from the parotid gland.

On March 25, 1927, almost 3 months after the beginning of the experiment, the left submaxillary and parotid glands were removed from Guinea Pigs 10-61 and 10-65. The specific lesion was found in the sections prepared from the submaxillary gland of Guinea Pig 10-65. In those prepared from the parotid gland of this guinea pig an area of reaction was present, but no acidophilic nuclear inclusion bodies were found. The specific lesion was found neither in the submaxillary nor in the parotid gland of Guinea Pig 10-61.

On March 25, 1927, after the removal of the left submaxillary gland and the left parotid gland, Guinea Pigs 10-61 and 10-65 received an intracerebral inoculation of active guinea pig virus. Guinea Pig 10-61 succumbed on the 6th day and the histological examination of the brain showed a typical meningitic exudate with cells containing acidophilic nuclear inclusion bodies. Guinea Pig 10-65 remained well.

The problem of spontaneous infection in this disease is an extremely difficult one. In the experiment reported above, three of four guinea pigs became infected in periods varying from 1 to 3 months. The other guinea pig, though closely confined in the same cage with these animals, never developed the specific lesion in either the submaxillary or parotid glands, and remained susceptible to intracerebral inoculation of the guinea pig virus when more than 3 months of age. In certain other experiments it was thought possible to exclude the occurrence of spontaneous infections of the submaxillary gland, by using only guinea pigs of known age. In one instance, however, the submaxillary gland of a guinea pig 22 days old was found to be infected. There are apparently other factors besides those of age and exposure to the virus that determine whether or not the submaxillary glands of guinea pigs become infected with this disease.

Passive Immunity.—It has been impossible to demonstrate any virucidal properties in the sera of full grown guinea pigs which have been spontaneously infected, or in young guinea pigs which have developed the infection in the submaxillary glands as the result of subcutaneous injections. The failure to show virucidal action in the serum of immune animals in this disease can probably be attributed to the fact that the quantitative factors have not been adequately controlled in these experiments. Further work needs to be done on this phase of immunity.

In the first two experiments on this subject, equal quantities of guinea pig virus and the pooled serum from three full grown guinea pigs, which were subsequently shown to be carrying the infection in their submaxillary glands, were combined and allowed to stand at room temperature for 30 minutes and 0.2 cc. of the mixture was then injected intracerebrally into young guinea pigs. In the same way the serum of a very young guinea pig, 6 days old, whose submaxillary gland did not show the specific lesion, was combined with an equal quantity of guinea pig virus, and injected intracerebrally into young guinea pigs. As additional controls, young guinea pigs were also injected intracerebrally with 0.2 cc. of a mixture of equal quantities of virus and Locke's solution.

In these experiments, even when the dose of virus used was small, the sera of the full grown guinea pigs did not prevent death or the

formation of a meningeal exudate containing cells showing acidophilic nuclear inclusion bodies.

Transmission of the Guinea Pig Virus in Series; from Skin to Submaxillary Gland and from Submaxillary Gland to Skin.—The regularity with which the specific lesion appeared in the submaxillary glands following subcutaneous inoculation of the virus was so striking that it was thought that this might be an easy way to transmit the virus from guinea pig to guinea pig in series. In this experiment, only pigs of known age, which had been born on the premises, were used. In order to control the experiment as accurately as possible, each litter of guinea pigs was, whenever possible, divided into two lots. Two guinea pigs born in one litter were inoculated subcutaneously with an emulsion of virulent submaxillary glands from full grown guinea pigs. As a control two other pigs of the same litter were inoculated with an emulsion of the submaxillary glands obtained from a full grown rabbit. 2 weeks after the subcutaneous inoculation, the submaxillary glands were removed from the guinea pigs injected with guinea pig virus, a piece was taken from each gland for section, and the remainder of the glands emulsified and injected subcutaneously into other guinea pigs of known age. In the same way the submaxillary glands were removed from the two pigs which had been injected subcutaneously with rabbit submaxillary gland, a piece was taken for histological section, and the rest was emulsified and injected subcutaneously into guinea pigs of known age. After 2 weeks the same steps were repeated. In this way the virus was carried through seven generations, the submaxillary glands of the previous generation always being injected subcutaneously into the guinea pigs of the next generation. The control series has been continued exactly in the same way as the series started with the guinea pig virus. In order to determine the virulence of the material used for subcutaneous inoculation in each generation, intracerebral inoculations were also made. Young pigs inoculated intracerebrally with the submaxillary glands of the series started with guinea pig virus have become sick and, in many instances, died. Histological examination of the brains of these animals have shown a typical meningitic exudate containing cells with nuclear inclusion bodies. Guinea pigs inoculated intracerebrally with an emulsion of the submaxillary glands removed from

TABLE III.

Subc. inoc.	Inoculum	Date	G. P.	Litter	Age	Submax. gland	
						Section	Result
I generation					*days*		
0.1 cc.	Submax. glands from 2 old guinea pigs	Nov. 8	87-1	A	4	768b	Pos.
		Nov. 9	87-2	"	"		
			87-5	B	"		
Removal of glands		Nov. 22	87-1	A	18	838b	Pos.
			87-2	"	"	839b	Neg.
			87-5	B	"	840b	Pos.
II generation							
Subc. inoc. 0.1 cc.	87-1	Nov. 22	94-7	D	7		
	87-2	Nov. 23	94-8	"	"		
	87-5		94-9	E	"		
Removal of glands		Dec. 7	94-7	D	22	927b	Pos.
			94-8	"	"	928b	Pos.
			94-9	E	"	929b	Pos.
III generation							
Subc. inoc. 0.1 cc.	94-7	Dec. 7	47	G	2		
	94-8	Dec. 8	48	"	"		
	94-9		49	F	8		
Removal of glands		Dec. 21	47	G	16	983b	Pos.
			49	F	22		
			48*	G	16		
IV generation							
Subc. inoc. 0.2 cc.	47	Dec. 21	10-42	H	7		
	49		10-43	"	"		
Removal of glands		Jan. 5	10-42	H	22	1027b	Pos.
			10-43	"	"	1028b	Pos.

* No. 48 used for section only.

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.

Negative indicates that no lesions were found in the glands.

TABLE III—*Concluded.*

Subc. inoc.	Inoculum	Date	G. P.	Litter	Age	Submax. gland	
						Section	Result
					days		
V generation							
Subc. inoc. 0.2 cc.	10-42		10-75	J	4		
	10-43	Jan. 5	10-76	"	"		
			10-77	I	13		
Removal of glands			10-75	J	19		
		Jan. 20	10-76	"	"	1068b	Pos.
			10-77	I	28		
VI generation							
Subc. inoc. 0.2 cc.	10-75		11-06	K	5		
	10-76	Jan. 20	11-07	"	"		
	10-77						
Removal of glands		Feb. 3	11-06	"	19	1107b	Pos.
			11-07	"	"	1108b	Pos.
VII generation							
Subc. inoc. 0.2 cc.	11-06	Feb. 3	11-36	H	6		
	11-07		11-37	L	8		
Removal of glands		Feb. 17	11-36	H	20	1148b	Pos.
			11-37	L	22	1149b	Pos.

the animals of the control series have usually remained well. In certain instances, when these guinea pigs died of other causes, the histological examination of the brain proved negative. The sections of the submaxillary glands of the guinea pigs of the series started with guinea pig virus have in every instance, except one, shown the specific lesion. On the other hand sections from the glands of the first six animals of the control series were all negative. The seventh animal of the series, however, Guinea Pig 11-41, though only 22 days old, developed a spontaneous infection. The guinea pigs in the control series were kept together in a separate cage, but no special precautions, such as sterilizing the cage, were taken. The guinea pigs were allowed

TABLE IV.

Control Series Started with Rabbit Submaxillary.

	Inoculum	Date	G. P.	Litter	Age	Submax. gland	
						Section	Result
I generation					*days*		
Subc. inoc. 0.1 cc.	Rabbit		87-3	A	4		
		Nov. 2	87-4	"	"	769b	Neg.
		Nov. 9	87-6	B	"		
			87-7	"	"		
Removal of glands			87-3	A	19	841b	"
		Nov. 23	87-4	"	"	842b	"
			87-6	B	"	843b	"
			87-7	"	"	844b	"
II generation							
Subc. inoc. 0.1 cc.	87-3	Nov. 23	95-2	E	8		
	87-6	Nov. 24	95-3	D	"		
	87-7		95-4	E	"		
Removal of glands		Dec. 7	95-2	E	22	930b	Neg.
			95-3	D	"	931b	"
			95-4	E	"	932b	"
III generation							
Subc. inoc. 0.1 cc.	95-2	Dec. 7	52	G	2		
	95-3	Dec. 8	53	F	8		
	95-4		54	"	"		
Removal of glands		Dec. 21	53	"	22	984b	Neg.
			54	"	"		
IV generation							
Subc. inoc. 0.2 cc.	53	Dec. 21	10-44	H	7		
	54		10-45	"	"		
Removal of glands		Jan. 5	10-44	"	22	1029b	Neg.
V generation							
Subc. inoc. 0.2 cc.	10-44	Jan. 5	10-81	I	13		
			10-82	"	"		
			10-80	J	4		
Removal of glands		Jan. 20	10-81	I	28	1069b	Neg.
			10-82	"	"		
			10-80	J	19		

Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.

Negative indicates that no lesions were found in the glands.

TABLE IV—*Concluded.*

	Inoculum	Date	G. P.	Litter	Age	Submax. gland	
						Section	Result
					days		
VI generation							
Subc. inoc. 0.2 cc.	10-81, 10-82, 10-80	Jan. 20	11-08	K	5		
Removal of glands		Feb. 3	11-08	"	19	1109b	Neg.
VII generation							
Subc. inoc. 0.2 cc.	11-08	Feb. 3	11-40	M	6		
			11-41	L	8		
Removal of glands		Feb. 17	11-40	M	20	1150b	Neg.
			11-41	L	22	1151b	**Pos.**

to remain with their mothers for 1 week after birth. It is possible that, if these factors had been more carefully controlled, the spontaneous infection of Guinea Pig 11-41 might not have occurred. The results of these experiments are shown in Tables III and IV.

Transmission of the Guinea Pig Virus from Submaxillary Gland to Submaxillary Gland.—The guinea pig virus has also been transmitted through seven generations in series by direct inoculation from gland to gland.

In this experiment guinea pigs of known age were not available. In every instance one submaxillary gland was removed at the beginning of the experiment and prepared for histological examination. In the first generation, Guinea Pig 10-10 was given a large injection of guinea pig virus subcutaneously. The left submaxillary gland was removed 12 days after inoculation, a small piece was prepared for histological section, and the rest emulsified and used for injection. In all subsequent generations injections were made directly into the left submaxillary gland. 14 to 17 days following inoculation, the injected submaxillary gland was removed, a piece prepared for histological section, and the rest placed in 50 per cent glycerol until cultures could be made to exclude the possibility of

TABLE V.

G. P.	Removal of gland at beginning of experiment						Removal of other gland					
	Gland	Date	Section	Result	Inoculum	Site	Gland	Date	Day	Section	Result	Time in glycerol
I generation												
10-10	Right	Dec. 8	945b		0.7 cc. virus	Subc.	Left	Dec. 20	12	980b	**Pos.**	24 hrs.
II generation												
10-48	Right	Dec. 21	985b	Neg.	Left submax. G. P. 10-10	Left submax.	Left	Jan. 4	14	1024b	**Pos.**	24 hrs.
10-49	"	"	986b	"		" "	"	"	"	1025b	**Pos.**	"
10-50	"	"	987b	"		" "	"	"	"	1026b	Neg.	"
III generation												
10-66	Right	Jan. 5	1030b	Neg.	Left submax. G. P. 10-48, 10-49, 10-50	Left submax.	Left	Jan. 22	17	1071b	Neg.	6 days
10-68	"	"	1032b	"		" "	"	"	"	1072b	**Pos.**	"
10-69	"	"	1033b	"		" "	"	Jan. 17	12	1056b	**Pos.**	"
10-70	"	"	1034b	"		" "	"	Jan. 22	17	1073b	**Pos.**	"
10-71	"	"	1035b	"		" "	"	"	"	1074b	**Pos.**	"
IV generation												
11-24	Right	Jan. 28	1093b	Neg.	Left submax. G. P. 10-66, 10-68, 10-70, 10-71	Left submax.	Left	Feb. 14	17	1139b	**S. pos.** P. neg.	0
11-25	"	"	1094b	"		" "	"	"	"	1140b	**S. pos.** **P. pos.**	0

V generation												
4a	Right	Feb. 14	1141b	Neg.	Left submax. G.P. 11-24, 11-25	Left submax.	Left	Mar. 1	15	5c	S. pos. P. neg.	24 hrs.
5a	"	"	1142b	"		" "	"	"	"	6c	S. pos. P. pos.	"
VI generation												
49a	Right	Mar. 2	8c	Neg.	Left submax. G.P. 4a, 5a	Left submax.	Left	Mar. 16	14	45c	S. pos. P. neg.	24 hrs.
51a	"	"	10c	"		" "	"	"	"	46c	S. " P. "	"
VII generation												
32-3	Right	Mar. 17	47c	Neg.	Left submax. G.P. 49a, 51a	Left submax.	Left	Apr. 2	15	79c	S. pos. P. neg.	
32-4	"	"	48c	"		" "	"	Mar. 24	7	67c	S. pos.	

Left submax. = left submaxillary gland; S. = submaxillary gland; P. = parotid gland.
Positive indicates that acidophilic nuclear inclusion bodies were found in the duct cells of the glands.
Negative indicates that no lesions were found in the glands.

bacterial contamination. The gland was then washed free of glycerol, emulsified, and used for injection into the next group of guinea pigs. In some cases, both the submaxillary gland and the parotid gland were examined histologically to see if the infection had spread from the submaxillary gland to the parotid.

None of the right submaxillary glands removed before the inoculation of the left submaxillary gland showed the specific lesion. The great majority of the injected glands showed acidophilic nuclear inclusion bodies and were virulent when inoculated into the brains of young guinea pigs. In one instance, both the submaxillary gland and the parotid glands showed the specific lesion, in four others the nuclear alterations were found in the submaxillary gland only.

Summary of Transmission Experiments.—The experiments recorded in Tables III to V indicate fairly conclusively that the guinea pig virus can be transmitted in series. The striking characteristic of this virus is its marked predilection for glandular tissue, which accounts for the fact that it was impossible to transmit the virus from brain to brain.

Is the Guinea Pig Virus Related to "Kurloff Bodies"?—The occurrence of peculiar structures, known as "Kurloff bodies," in the cytoplasm of the mononuclear cells of guinea pigs' blood was first observed by Kurloff (3) in 1888. Although many investigators have studied the "Kurloff bodies," it has been impossible to draw any definite conclusions as to their nature. Some workers maintain that they represent vacuoles, others that they are true inclusion bodies belonging to the "Chlamydozoa." Their infectious nature, however, has never been proved. All attempts to transmit them from one animal to another have failed. It is of interest that "Kurloff bodies" are not present in the blood of guinea pigs at birth. According to Senez (4) they appear as early as the 7th day; Bender (5), on the other hand, has never observed them in guinea pigs less than 1 month old. The absence of "Kurloff bodies" in very young guinea pigs and the development of these structures in the blood of the majority of full grown guinea pigs, suggested that there might be some relationship between them and the infection of young guinea pigs with guinea pig virus. The fact, however, that "Kurloff bodies" always lie in the cytoplasm of the mononuclear cells, whereas the lesion of the guinea pig virus is characterized by nuclear inclusion bodies, indicates that there

is probably no relation between the two conditions. In order to exclude this possibility, citrated blood of a full grown guinea pig containing "Kurloff bodies," was injected intracerebrally into a young guinea pig. The animal remained well. On the 6th day it was killed and sections prepared from the brain. No meningitic exudate or other cerebral lesion was found. In order to obtain a greater concentration of mononuclear cells containing "Kurloff bodies," 10 cc. of sterile broth was inoculated into the peritoneal cavity of a full grown guinea pig. The following day the peritoneal exudate was examined for the presence of cells containing "Kurloff bodies." A fair number of these cells were found to be present. The cells were washed with salt solution and injected intracerebrally into two young guinea pigs. These animals also remained well.

CONCLUSIONS.

1. It has been shown that the guinea pig virus localizes in the submaxillary glands of young guinea pigs following subcutaneous, intraperitoneal, or intravenous injection of active material, and that the specific lesion is demonstrable in the glands in 12 to 15 days. When an active infection of the gland has been produced in this way, the guinea pigs are refractory to intracerebral inoculation of the virus.

2. No lesion develops in the submaxillary glands of young guinea pigs injected subcutaneously with guinea pig virus which has been inactivated by heat. Young guinea pigs which have received injections of heat-killed virus do not become refractory to intracerebral inoculation of the virus.

3. When young guinea pigs from which both submaxillary glands have been removed are injected subcutaneously with active virus, the virus localizes in the parotid gland, and the animals become refractory to intracerebral inoculation.

4. It has been impossible to demonstrate virucidal properties in the sera of adult guinea pigs which have become spontaneously infected with the virus, or in the sera of young guinea pigs which have been artificially rendered refractory to intracerebral inoculation.

5. It has been possible to transmit the virus from guinea pig to guinea pig continuously in series through seven animals by direct inoculation from submaxillary gland to submaxillary gland.

6. The fact that the virus regularly localizes in the submaxillary glands following subcutaneous inoculation has been utilized in passing the virus from guinea pig to guinea pig. 2 weeks after the subcutaneous inoculation of the virus into young guinea pigs, the active agent was present in the submaxillary glands. Emulsions of the submaxillary glands of these animals were then used for the subcutaneous injection of another group of young guinea pigs. In this way the virus was transmitted continuously from skin to submaxillary gland through a series of seven animals.

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STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

IV. Effects of Certain Physical and Mechanical Factors on Extracorporeal Thrombosis with and without the Use of Anticoagulants.

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It is well known that coagulation of drawn blood may be retarded experimentally by cold, and that moderate heating hastens coagulation. In fact, it has been claimed by some (Starling) that platelets increase in numbers in cold blood and that they can actually be "manufactured" by cooling platelet-free plasma. In this connection I have no evidence to offer, but it is important to know the influence of heat and cold on clot formation in the circulating blood. This may have some bearing on matters of treatment; consequently some experiments have been carried out by the method already described for the study of extracorporeal thrombosis.

Effects of Temperature.

Cold.—Rabbits weighing about 2 kilos were anesthetized and operated on as already described. The apparatus, filled with physiologic solution of sodium chloride was attached to the carotid artery and jugular vein, the collodion tube being immersed in cool physiologic sodium chloride solution (5°C.). The clamps were detached and the blood began to circulate very violently through the apparatus. In spite of the container being surrounded by ice, the cool solution in the container became warm (10° to 15°) very soon, as it was influenced by the warm arterial blood. To keep the collodion tube cool the solution in the container was changed many times.

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On marked cooling, the blood in the apparatus continued to flow for from 15 to 25 minutes, while at body temperature it ceased in from 4 to 10 minutes. Finally the circulation was stopped by the obstruction of the arterial cannula (rarely of the venous cannula) by white thrombi. Before obstruction was complete the blood current became slow and white thrombi appeared in several places in the apparatus, especially in the arterial tubes and to some extent in the collodion tube. Still circulation sometimes went on. Gradually the pulsation of the collodion tube and jugular vein became less marked, and mixed thrombi appeared in the venous cannula, and extended into the vein. When the apparatus was detached, as soon as the obstruction occurred, the blood in the arterial cannula and in the arterial half of the apparatus was already clotted and mixed thrombi appeared on the irregular surface of the connecting parts of the glass tubes, while about half of the blood in the venous half of the apparatus still remained fluid. In the collodion tube the blood was clotted in laminæ, a part of the blood still remaining fluid; a few small white thrombi were sometimes seen also.

From this it is clear that low temperature tends to retard clotting in the artificial loop and consequently the occurrence of obstruction is postponed. Nevertheless, white thrombi were observed in several parts of the apparatus.

Heat.—In two experiments the effects of heat (40°C.) were investigated. After a few minutes circulation, fibrin formed on the abnormal surface of the apparatus, especially in the collodion tube and venous cannula. Obstruction of the venous cannula by red clot occurred after from 4 to 6 minutes circulation. Soon after obstruction, all the blood in the apparatus clotted as a solid column; lamination was not marked. The formation of white thrombi was scarcely evident.

The results of heat (55°C.) applied around the collodion tube are much more interesting for they indicate the rapidity with which clotting may occur. After from 2 to 6 minutes the venous cannula was totally obstructed and a solid column of blood was taken out of the apparatus. In serial sections only coagulated blood was seen, instead of the characteristic structure of red and white thrombi. Rapid coagulation does not allow sufficient time for stratification or collection of platelets.

Effect of Mechanical Factors Such as Compression and Obstruction of Vessels.

When the blood is normally coagulable and the carotid artery is partially compressed and the stream retarded the arterial cannula is soon obstructed by white thrombi, mixed thrombi form in the artery, and the entire blood in the apparatus comes to a standstill and quickly clots as a solid mass.

Partial Occlusion of the Carotid Artery.—The results in the following protocol are typical of the findings in three experiments.

At 2.25 p.m. Rabbit 28, weighing 1,800 gm. was injected intravenously with 30 mg. of heparin. At the same time the apparatus was attached and the carotid artery compressed with metal clip so that the blood flowed at the rate of 30 drops a minute. Clotting time was more than 30 minutes.

At 2.55 pulsation was recognizable. A white thrombus obstructing the arterial cannula was removed. With a new set-up, the experiment was continued, the blood flowing at the rate of 30 drops a minute.

At 3.20 the stream stopped again. The collodion tube was cut off. The constricted part of the arterial cannula was filled with white thrombi and totally obstructed. There was a big red thrombus (6 mm. long, 4 mm. wide and 3 mm. thick) in the bottom of the arterial end of the collodion tube, and in the arterial half of the apparatus were many white thrombi, which were covered by sedimented erythrocytes.

In spite of the incoagulability of the blood, the slowing of the blood stream has a tendency to facilitate the formation of red thrombi on the foreign lining of the apparatus and, on account of the marked retardation of the stream, the thrombi are chiefly formed in the arterial half, in the collodion tube at the arterial inlet just as in the case of Aschoff's sand banks. Obstruction of the arterial cannula by a mass of white thrombi is easily demonstrated.

In the serial sections this thrombus from the collodion tube is built on a base of very tiny white thrombi and is composed of red clots, in which wide fibrin bands are interwoven. Despite the incoagulability erythrocytes easily precipitate and fibrin is formed rather quickly.

Complete Obstruction of the Carotid Artery.—In two experiments with anticoagulant injection* a few minutes after the circulation through the apparatus was established, the carotid artery was obstructed by a clamp. The apparatus was detached 15 minutes and 50 minutes respectively, afterwards. There was a mixed thrombus

* With larger or continuous dose of heparin clotting might be entirely prevented.

in the carotid artery between clamp and cannula which followed the white thrombus of the constricted part of the arterial cannula. In the latter, also in the venous cannula, in the jugular vein and here and there in the other parts of the apparatus, blood clot adhered to the wall. In the collodion tube the greater part of the blood remained fluid but, in the bottom, thin layers of red clots and one big mixed thrombus adhered to the wall of the arterial half of the collodion tube near the inlet and another was present at the outlet.

In the case of partial obstruction of the carotid artery the blood stream in the apparatus is at a standstill and further growth of white thrombi is very much reduced because no new blood platelets or leucocytes will be brought in contact with the wall.

Obstruction of the Jugular Vein.—The findings in the following are characteristic of those in four experiments.

At 2.30 p.m. Rabbit 44, weighing 1,850 gm. was injected with 40 mg. of heparin intravenously. At 5.05 the apparatus was attached and marked stream and pulsation were observed in it. At 5.15 the jugular vein was obstructed by a clamp. Then the blood stream in the arterial cannula was violent and the pulsation of the collodion tube still marked; soon afterwards the blood in the venous tubes and in the collodion tube became dark, but the pulsation in the arterial half persisted.

At 5.45, 55 minutes after connection, the clotting time was longer than 30 minutes; pulsating movement in the arterial cannula was still marked. 1 hour after the connection the apparatus was detached. In the apparatus white thrombi were not numerous. Sedimentation of erythrocytes occurred in the venous corner tube. The collodion tube contained at its arterial end a large mixed thrombus, consisting mainly of large clumps of platelet thrombi; about twenty clumps were connected by fibrin and erythrocytes.

SUMMARY.

The effects of environmental temperature on thrombosis in circulating blood were tested in the extracorporeal loop by the method previously described. Cold (7° to 15°C.) about the collodion tube retards the clotting of circulating blood. Obstruction of the arterial cannula occurs after from 15 to 25 minutes. Heat (40° and 55°C.) tends to hasten clotting; obstruction by red clot takes place after from 4 to 6 minutes and from 2 to 4 minutes, respectively. In the latter case no characteristic thrombus structure may be seen. These results were what might be expected. The formation of white thrombi

occurs even under the influence of cold, and they continue to form and grow as long as the circulation continues.

When the carotid artery is partially occluded and the blood stream slowed, after the injection of the anticoagulant, the blood has a tendency to form red thrombi on the foreign surface of the vascular loop. The thrombi are deposited chiefly in the arterial half of the apparatus, especially at the bottom of the arterial end of the collodion tube, just as in the case of Aschoff's sand experiments. In spite of incoagulability of heparinized blood, red clots, with interwoven fibrin bands, are found. Also very tiny white thrombi may form in other parts of the collodion tube.

The effect of complete obstruction of the carotid artery and jugular vein on thrombus formation was studied after the use of anticoagulant. When the carotid artery is obstructed the formation of white thrombi is negligible and sedimentation of erythrocytes and deposition of fibrin appear throughout the apparatus; then the blood, at a standstill, clots very slowly.

When the jugular vein is obstructed, dislodgment of platelet thrombi probably results from the pulsating movement, and sedimentation of erythrocytes follows, forming mixed thrombi. The dislodged white thrombi tend to gather in the bottom of the tubes of the widest caliber, especially in the collodion tube.

STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

V. Influence of Certain Chemical Substances on Extracorporeal Thrombosis with Special Reference to the Efficacy of a Combination of Heparin and Magnesium Sulfate.

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With the method of studying experimental extracorporeal thrombosis already described, the influences of certain chemical substances, such as distilled water, bile salts, *d*-glucose, urea and calcium chloride, have been examined.

The Effect of Distilled Water.

It is well known that shed or hemolyzed blood, when injected into the circulation, may cause the liberation of fibrin and subsequently intravascular clotting. On the other hand, it has been stated by Reed that heparinized blood, despite its incoagulability, can clot very soon after the addition of distilled water. In this connection, I have carried out a series of experiments and have verified this fact, as will be seen from the results.

Experiment 1.—A rabbit weighing 2100 gm. was given 50 mg. of heparin (Hynson, Westcott and Dunning) intravenously. Blood was drawn immediately from the carotid artery into a paraffined vessel; 1 cc. of the blood was put into each of a series of small test-tubes and various amounts of distilled water were added to each and clotting time was recorded (Table I). Accordingly I attempted to see what the effect would be of distilled water placed outside of the collodion membrane.

Experiment 2.—When the collodion tube of the circulatory loop was surrounded by distilled water, the latter passed through the membrane into the circulation, causing, from 5 to 10 minutes later, hemolysis of the heparinized blood flowing along the wall of the collodion tube. 30 minutes or more later, fibrin formed, and

coagulation of hemolyzed blood followed. In the serial sections this clot looked almost entirely homogeneous, and neither structure nor formed elements could be differentiated because of hemolysis.*

Two reasons are advanced to explain this phenomenon: one that tissue extract or fibrinogen and hemoglobin set free by hemolysis neutralize the heparin action, and the other, dilution of the anticoagulant. According to Howell, the latter explanation is less probable.

TABLE I.

Clotting Time of Heparinized Blood in Water.

	5 min.	7 min.	12 min.	20 min.	40 min.	60 min.	18 hrs.
1 cc. of heparinized blood in 0.1 cc. water	−	−	−	+	+	++	++
1 cc. of heparinized blood in 0.2 cc. water	±	+	+	+	+	++	++
1 cc. of heparinized blood in 0.5 cc. water	+	+	+	++	+++	+++	+++
1 cc. of heparinized blood in 1.0 cc. water	+	+	+	++	+++	+++	+++
1 cc. of heparinized blood in 2.0 cc. water	−	−	−	+	+	+	++
1 cc. of heparinized blood in 3.0 cc. water	−	−	−	−	−	±	±
Control	−	−	−	−	−	−	−

The Influence of Bile Salts on Clotting in the Heparinized Animal.

For a long time it has been known that jaundice tends to disturb the clotting process of the blood. This fact was also verified by Minot and his associates, who found, by using Howell's recalcification method, that the coagulation time was increased in a series of cases of jaundice. Haessler and Stebbins demonstrated that the clotting time of the blood, to which bile salts have been added, is proportional to the quantity of bile salts present and that the bile interferes with the conversion of fibrinogen into fibrin and not with the formation of

* It will have been observed that the tenacity of both red and white thrombi has varied under different experimental conditions. This may depend on variation in the character of the agglutination of the elements or on the amount and nature of the fibrin deposited. Since thrombi *in vivo*, under different conditions, show similar variation in tenacity, the relation of these phenomena to embolism assumes prime importance. Pathologically the question is not only why thrombi form, but why emboli are dislodged in some cases and not in others. In this connection it is to be remembered that fibrin deposited from fibrinogen *in vitro* by any other than biologic methods is not crystalline (Howell).

thrombi. To my knowledge, no one has yet investigated *in vitro* or *in vivo* the influences of bile salts on heparinized blood. I carried out some experiments with a rabbit, whose blood had been rendered less coagulable by intravenous injections of heparin, also some *in vitro* with the blood of normal rabbits, to which heparin was added (16 mg. to each 40 cc.) and found that in both instances more than 1 drop of 1 per cent solution of bile salts to each cc. of blood caused hemolysis, but no decrease of the clotting time.

Experiments were carried out by means of the extracorporeal loop after the injection of heparin. Bile salts, dissolved in physiologic sodium chloride solution (1 or 2 gm. for each 100 cc.) were placed in the container surrounding the collodion tube of the apparatus, and also, in other cases, introduced directly into the circulatory loop through the rubber tube connecting the arterial cannula and the side tube. Thus the simultaneous influence of bile salts and of heparin on experimental thrombosis was observed.

In the former case, that is with bile salts in the dialyzing container, so long as no coagulation or no obstruction occurred the white thrombi not only appeared in abundance in the venous half of the apparatus (the glass tube, paraffined part and collodion tube) but they grew more rapidly than when physiologic sodium chloride solution was used. Radiation, or fan formation, was less pronounced. In the structure of this thrombus, the platelets, of course, play a great part, but the rôle of the leucocytes as a constituent of the thrombus should not be overlooked. The size of each white thrombus at the end of 1 hour or 3 hours was many times larger than when physiologic sodium chloride solution was used for a corresponding period. They appeared as cauliflower-like excrescences.

The thrombi obtained after injection of bile salts were also unquestionably larger than when physiologic sodium chloride solution alone was used. Furthermore, the white thrombi appeared in the arterial half of the apparatus, especially near the place of injection, in the arterial corner tube and in the arterial end of the collodion tube, despite the speed of the stream. The arterial corner tube was nearly obstructed after 3 hours, in spite of the fact that its lumen was many times greater than that of the narrow part of the cannula.

In both cases, when the solution of bile salts was placed outside of

the collodion tube and also when it was injected into the circulation, the mass of white thrombi was loose, soft and fragile, being easily broken up into the individual platelet thrombi. There appeared to be less contractility, suggesting that fibrin was absent or if present was abnormal or of a poor quality. Even when complete obstruction

TABLE II.

Size and Number of White Thrombi with 1 or 2 Per Cent Bile Salts Placed Outside of the Collodion Tube.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			*mm.*	*mm.*		
Mar. 22	9	3 hrs.	0.60	0.50	2	No obstruction
			0.50	0.50	3	
May 6	22	3 hrs.	1.40	1.20	2	
			0.50	0.40	5	
			0.08	0.10 or	7	
			0.40	0.20	8	
			0.20	0.20	5	
May 13	25	4 hrs., 20 min.	1.40		Many	Long ranges of hillocks. No obstruction because the white thrombi gathered mostly in the wide part of the apparatus in the corner tube
June 28	52a	3 hrs.	0.10	0.08	More than 20	Two ranges of hillocks
			0.20	0.20		
			1.0	0.8		
June 28	52b	2 hrs., 30 min.	3.0	4.0		Large white thrombus in the corner tube obstruction
			0.06	0.08		

by a thrombotic mass occurred, no clotting followed. These phenomena are in part due to the action of the heparin, but it seems reasonable to consider that the bile salts also disturb the coagulation of heparinized blood, since such extreme results were not found with heparin alone.

The fact that white thrombi are readily stimulated by bile salts and the red thrombi formed through the influence of bile salts are fragile, might explain why postoperative pulmonary embolism is found in a relatively large number of cases of hepatic disease and gall stones (Wilson).

Typical protocols from a series of experiments follow, and the results of the series are shown in Tables II and III.

Experiment 3 (*Bile Salts by Dialysis*).—Rabbit 52a weighing 1800 gm. was injected intravenously with 40 mg. of heparin at 12.20 p.m. At 12.40 p.m. the apparatus was connected and 2 per cent bile salts in physiologic sodium chloride solution put outside the collodion tube. The blood stream was very rapid, pulsation of the collodion tube and the jugular vein being marked. The clotting time was more than 30 minutes. At 3.40 p.m. the collodion tube was removed. Throughout the whole apparatus many rather large white thrombi were seen, most of them confluent and constituting an inner coat or cast on the inner surface of the tubes. The individual thrombi were connected by abnormal appearing fibrin threads. In the collodion tube, similar conditions prevailed. There were innumerable small thrombi but a few were very large. The smaller types fused together to form the thick membrane composed of platelet clumps with numerous leucocytes. In one field more than twenty of the white thrombi measured 0.10 by 0.08 mm., and seven measured 0.20 by 0.20 mm. The large variety were many times larger than those observed in the case of physiologic sodium chloride solution in the heparinized animal. Microscopically large high hillocks of platelet clumps were seen with numerous leucocytes in them. The hillocks measured 1.0 by 0.8 mm., with sometimes a range of hillocks of 0.5 mm. width with valleys intervening.

TABLE III.

Size and Number of Thrombi with 2 Per Cent Solution Bile Salts Injected into the Vessel.

Date	No.	Time	Length	Width	No. in one field
1926		*hrs.*	mm.	mm.	
June 1	39a	3	1.00	0.80	3
			0.54	0.50	2
			0.40	0.40	3
June 1	39b	3	0.56	0.40	3
			0.44	0.40	3

Experiment 4 (Bile Salts Injected into the Arterial Side of the Apparatus).—Rabbit 39a weighing 1020 gm. was given 40 mg. of heparin intravenously at 11.30 a.m. At 12.00 the apparatus was connected and 3 cc. of 1 per cent solution of bile salts was injected into the arterial side of the extracorporeal loop. At 1.00 p.m. 1 cc. of solution of bile salts was injected in the same way. There was a violent stream and marked pulsation in the artificial vascular loop. At 2.00 p.m. 1 cc. of solution of bile salts was injected. At 3.00 p.m. the apparatus was detached; the blood in it remained incoagulable. In the arterial corner tube there were many white thrombi which obstructed the lumen. In the arterial end of the col-

TABLE IV.

Size and Number of White Thrombi with 1 or 2 Per Cent d-Glucose Solution Outside of the Collodion Tube.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			*mm.*	*mm.*		
May 7	23b	1 hr.	0.10	0.08	More than 20	In the figure of radiation
			0.04	0.04	More than 25	
June 21	48a	3 hrs.	0.26	0.024	4	
June 21	48b	3 hrs.	0.20			Irregular ranges of hillocks
			0.40	0.40		
			0.20	0.20	Many	Radiation
June 21	48c	2 hrs.	0.10	0.10	12	Radiation
July 19	61a	2 hrs.,	0.24	0.10	6	
		30 min.	0.12	0.10	5	
July 19	61b	30 min.				Blood coagulable
July 21	65	2 hrs.	0.16	0.16	6	Radiation
			0.10	0.08	4	

lodion tube a good many large white thrombi had gathered, almost obstructing it. Also, in the outer lower part and bottom of the collodion tube there were numerous rather large white thrombi, each hillock being high. Fern-like radiating figures were also seen. In one field, two or three white thrombi measured 1.00 by 0.88 mm., two measured 0.54 by 0.50 mm. and three measured 0.40 by 0.40 mm.

Influence of d-Glucose.

Changes in blood sugar content may cause only slight fluctuations in the clotting time of the blood. It was desirable to investigate the

influence of *d*-glucose on thrombosis, because there is a high blood sugar content, not only in diabetes, but also after *d*-glucose has been introduced as a therapeutic measure. The essential data from twelve experiments on the effect of *d*-glucose on heparinized blood ***in vivo*** are presented. When *d*-glucose dissolved in physiologic sodium chloride solution (1 or 2 gm. for each 100 cc.) was placed in the container outside of the collodion sac, numerous tiny white thrombi appeared in the course of time (Table IV). The size of each at any given time was relatively smaller than those seen with physiologic sodium chloride solution in the container. The thrombi in the

TABLE V.

Size and Number of Thrombi with 10 Per Cent Glucose Injected into the Vessel.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			*mm.*	*mm.*		
June 23	50a	3 hrs.	0.08	0.04	1 or 2	
June 26	51a	3 hrs.	0.08	0.06	9	
June 26	51c	3 hrs.	0.08	0.06	1 or 2	
June 26	51b	30 min.	No thrombi			Obstruction of carotid artery
June 23	50b	1 hr., 5 min.	6.0	3.0		Red cell clumps and clot

collodion tube tended to assume radiating figures in the majority of cases. They were composed chiefly of platelets, and, on and around them, there were in the earlier period a very few leucocytes but in the later stages a considerable number. Even in an experiment of three hours duration obstruction and consequent stopping of the stream was rare, even though, in the constricted part of the cannula, and under certain conditions elsewhere, the thrombi congregated, making rather a loose mass which could be broken easily; fibrin formation seemed to be delayed and abnormal or poor, while with physiologic sodium chloride solution, despite ample action of the heparin, fibrin did appear in rather a short time, and contributed to the obstruction.

Despite these changes, the *d*-glucose did not appear to shorten the clotting time of heparinized blood.

When the *d*-glucose solution (10 per cent) was injected into the artificial circulatory loop through the gum tube between the arterial cannula and side tube, white thrombus formation was rather rare throughout the apparatus (Table V). These findings suggest that hypertonic sugar solution tends to make platelets and leucocytes conglomerate less readily; hence the thrombotic elements do not precipitate readily nor adhere to the vessel wall so that they are carried away. These results were constant for the series of experiments although they are difficult to explain at present.

Influence of Calcium Chloride.

The exact significance of calcium in fibrin formation is still unsettled. Blood from which the calcium has been precipitated by oxalate or citrate will not coagulate, but the addition of calcium salts will promptly cause it to coagulate. As to the influence of calcium on blood clotting, the hypothesis usually accepted is that calcium ions are necessary for the transformation of prothrombin into thrombin. Howell considers that no kinase is necessary, the calcium activating the prothrombin whenever it is not inhibited by heparin. On the other hand, it is known that the presence of calcium in high concentration in the blood prolongs the coagulation time (Freund, Rosenmann and Loewenstein, and others).

Furthermore, Howell and Holt state that no amount of calcium causes clotting of heparinized blood, showing that the heparin does not act by decalcifying the blood. Considering the various rôles of calcium, such as the calcification of vessels, the field of blood clotting, or its wide intravenous use therapeutically, it is of considerable importance to investigate the influence of calcium on extracorporeal thrombosis. Therefore, eight experiments were performed after the preliminary injection of heparin.

Experiment 5.—Rabbit 26 weighing 1950 gm. was given 40 mg. of heparin intravenously at 1.30 p.m. The apparatus was connected at 1.40 p.m. and the collodion tube surrounded with 1 per cent of calcium chloride in physiologic sodium chloride solution, kept at body temperature. The blood stream had been kept vigorous by twice removing the obstruction of the venous cannula. At

TABLE VI.

Size and Number of Thrombi with 1 Per Cent Solution of Calcium Chloride Placed Outside of the Collodion Tube.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926			*mm.*	*mm.*		
Mar. 11	4a	4 hrs.	Small, white thrombi covered by red clot			Within 30 min. obstruction and sedimentation (4 hrs. and 20 min. after 50 mg. heparin injection); clotting time 5 min.
Mar. 12	5	2 hrs., 10 min.	0.30 0.16	0.24 0.12	5 8	Obstruction one time; removed Fibrin network
Mar. 15	6a	30 min.	0.16 0.10	0.14 0.06	2 15	
Apr. 25	13b	30 min.	0.10 0.08	0.06 0.06	2 3	
May 15	26	3 hrs.	5.00 2.40 1.20 0.40 0.14	1.5 1.20 1.00 0.30 0.12	1 1 4 4 20	Thrombus 1 mm. thick. Venous cannula cleaned up twice as obstruction occurred

TABLE VII.

Size and Number of Thrombi with Hourly Injections of 2 Per Cent Solution of Calcium Chloride.

Date	No.	Time	Length	Width	No. in one field	Remarks
1926		*hrs.*	*mm.*	*mm.*		
June 4	41a	3	1.10 0.16	0.40 0.12	4 15	
June 4	41b	3				All over the apparatus white thrombi causing obstruction, followed by red thrombi. 6 hrs. and 30 min. after 50 mg. heparin (1600 gm. body weight) injection, the clotting time was 5 min., but 20 min. later it was 2 min.

4.40 p.m. the stream was still violent; the clotting time *in vitro* was more than 30 minutes. The apparatus was disconnected. White thrombi in the tubes were very large and numerous (Table VI), especially in the venous half of the apparatus. The venous corner tube was thickly coated. The collodion tube contained large white thrombi in its venous half, while there were very few in the arterial half.

Experiment 6.—Rabbit 41a weighing 1600 gm. was given intravenously 50 mg. of heparin at 11.00 a.m. At 11.20 the whole apparatus was connected, and the collodion tube surrounded with physiologic sodium chloride solution, kept at body temperature; 2 cc. of 2 per cent solution of calcium chloride was injected very slowly into the annular circulation through the rubber tube, which unites the arterial cannula and the arterial side tube; after that 1 cc. of calcium solution was injected every hour in the same way. By 11.40 a.m., from the place of the injection down to the venous corner tube, numerous white thrombi had appeared; the stream was still vigorous. At 2.40 p.m., after 3 hours, the blood remained non-coagulable; the circulation had not been impeded. 3 hours after connection, the apparatus was detached; in the paraffined tubes a goodly number of large white thrombi adhered to the inner wall, making a thick coat (Table VII). Nevertheless, there remained a tiny tunnel through which the blood had been steadily circulating. In the collodion tube, white thrombi were connected with one another irregularly.

When the collodion tube of the apparatus is surrounded by calcium chloride in physiologic sodium chloride solution (1 or 2 gm. calcium chloride to 100 cc. physiologic sodium chloride solution) and at body temperature, the white thrombi appear chiefly in the venous half of the apparatus (the paraffined part of the glass tube and the collodion tube), and as long as the incoagulable blood flows, thrombi grow rather more rapidly than when physiologic sodium chloride solution is in the container; and the narrow part of the venous cannula is easily obstructed by masses of white thrombi, made up of clumps of platelets and leucocytes, interwoven by fibrin threads; the thrombi are not so fragile as when bile salts or *d*-glucose solutions are used. Sedimentation of erythrocytes readily follows, and fibrin seems to form easily. When the obstructing plug of the narrow part is removed as formed, the thrombi in the venous corner tube and collodion sac develop more speedily than in the case of sodium chloride solution and in the course of 2 or 3 hours the lumen of the corner tube which is many times wider than that of the narrow part of the cannula, is nearly obstructed by a thick coat. Otherwise on account of the prompt plugging of the narrow part, a further supply of hematogenic throm-

botic elements is lacking. As a result in the collodion tube, small white thrombi form, which are almost always covered by sedimented erythrocytes. Similar findings are encountered when 2 per cent calcium chloride solution is injected into the circulation.

Effects of Magnesium Sulfate on Extracorporeal Thrombosis.

The foregoing experiments probably shed some light on the problem of thrombosis; however, the ultimate object of these investigations was the prevention of thrombosis, especially of postoperative pulmonary embolism. For this purpose no special prophylactic methods are commonly adopted. On the basis that the slowing of the blood stream probably constitutes one of the most important causes, exercise and movement of the extremities, and massage have been advised by some in order to accelerate the rate of circulation. Similarly, desiccated thryoid has been employed by Walters.

Mason's attempts to protect experimentally against intravascular clotting with heparin are praiseworthy. But, as my numerous experiments show, the formation of white thrombi on the pathologic lining of the vessels was not prevented by a single dose of the heparin employed. The experiments with the injection of hypertonic *d*-glucose solution suggest that the introduction of hypertonic solution into heparinized blood will tend to combat white thrombus formation. Magnesium sulfate also comes into consideration. It has been employed by Fonio in an indirect method of counting blood platelets, to protect against the cohesive properties of the platelets. Some authors believe that there is a close relationship between the earlier stages of thrombosis and agglutinability of platelets. Recently, Hans Schulte investigated the influence of hydrogen ion concentration on agglutination of isolated blood platelets over a period of 12 hours, and reported that it is most marked at a pH of 4.3 to 3.5. He could recognize no influence of sodium chloride and calcium on the process. On the other hand, the results of my studies on experimental thrombosis show that calcium chloride solution seems to stimulate the appearance and development of white thrombi. One might expect some relationship between the presence of calcium chloride in the blood and the development of white thrombi. If, from the colloid-chemical viewpoint, the thrombus formation is closely related to the

agglutination of platelets as colloidal precipitation, magnesium ions and other bivalent positive ions should precipitate the platelets in the same way as calcium does. Thus the influence of magnesium sulfate on thrombosis would appear to be interesting and perhaps of considerable significance. One protocol will suffice.

Experiment 7.—Rabbit 38 weighing 1010 gm., was given 40 mg. of heparin intravenously at 10.25 a.m. At 11.10 a.m. the apparatus was connected and the collodion tube surrounded by 1 per cent magnesium sulfate in physiologic sodium chloride solution. During 3 hours there was a violent stream and marked pulsation in the collodion tube and jugular vein. Clotting time was more than 30 minutes. There was no clotting or obstruction. At 2.10 p.m. the apparatus was detached; there were very few tiny white thrombi in the arterial corner tube. In one place in the upper part of the venous half of the collodion tube an occasional tiny white thrombus was seen, which microscopically consisted of platelets; on each of the thrombi a few scattered leucocytes were recognized. Of twelve thrombi in one field, ten measured 0.08 by 0.04 mm., and two measured 0.10 by 0.06 mm.

From four experiments it was concluded that when the collodion tube was surrounded with 1 per cent solution of magnesium sulfate only a few small white thrombi appeared in the arterial half of the apparatus, while in the venous half very few or no white thrombi were found even when the rate of flow was slow enough to favor adequate formation of thrombi. In the collodion tube, which is the lowest and widest part of the circulatory loop, small white thrombi, often of microscopic size, were seen after 3 hours. The total number of such thrombi was very small in comparison with other experiments. Throughout a 3 hour experiment no obstruction had occurred, neither did any thrombi adhere to the glass cannulas. The action of magnesium sulfate on thrombosis is different from the action of calcium chloride. Magnesium appears to protect definitely against thrombosis, probably by the precipitation of small amounts of calcium as calcium sulfate.

The influence of the intravascular injection of magnesium sulfate solution was next examined. In two experiments the effect of the intravenous injection of magnesium sulfate solution (50 mg. for each kilo of body weight an hour) was tried without the use of any other anticoagulant. The coagulation time of the blood was increased from two to three times normal and thereby clotting in the artificial vascular loop was prevented for from 25 to 30 minutes. White thrombus formation was markedly reduced. Thin layers of blood clot were observed in the collodion tube, and mixed thrombi formed on the irregular surfaces of the apparatus and also in the jugular vein. The venous cannula, and a little later the arterial cannula, were finally obstructed by small masses of white thrombi and fibrin. Despite this, most of the blood in the loop remained fluid.

In the next set of six experiments, the intravenous use of a combination of magnesium sulfate and heparin yielded still more interesting results.

Experiment 8.—Rabbit 7 weighing 2250 gm., was given 20 mg. of heparin intravenously at 3.30 p.m. At the same time 1 cc. of 10 per cent magnesium sulfate was administered intravascularly into the ear vein. At 4.00 p.m. the apparatus was connected and the collodion tube surrounded by physiologic sodium chloride solution of body temperature. At 4.30 p.m. 1 cc. of 10 per cent magnesium sulfate solution was injected intravenously, and at 6.30 p.m. 1 cc. was again injected intravenously. At 7.00 p.m., after 3 hours experiment, the streaming was vigorous; no thrombi were recognized anywhere. The apparatus was detached. In the collodion tube and glass tubes no thrombi were found, even by microscopic examination. The clotting time of the blood was more than 30 minutes.

When a large quantity of 10 per cent magnesium sulfate solution was injected into the annular circulation, or introduced into the general circulation, after intravenous administration of heparin, no thrombi appeared for a certain period in any part of the apparatus. When physiologic sodium chloride solution was used around the outside of the collodion tube, without injection of magnesium solution, white thrombi appeared in 15 minutes in the venous corner tube; 2 cc. of 10 per cent magnesium sulfate solution to 2250 gm. of body weight protected against the occurrence of the first stages of thrombosis for a period longer than 3 hours; 3 cc. of the solution to 2100 gm. of body weight for longer than 6 hours. A few platelet clumps were formed despite the action of magnesium, and sometimes they appeared in the narrow part of the venous cannula, constituting a kind of dam. But when 1 cc. of 10 per cent solution of magnesium sulfate was injected every hour into the circulation of the rabbit, no thrombi appeared. Besides, throughout the experiments, neither obstruction of the circulation nor hindering of the action of heparin has been observed. 3 cc. of 10 per cent solution of magnesium sulfate to 2100 gm. of body weight appears to be almost a lethal dose. This toxicity is at any rate a weak point from the standpoint of clinical application, even though magnesium sulfate solution can prevent the first stages of thrombosis.

Effects of a Combination of Magnesium Sulfate and Bile Salts.—In order to see the antagonistic action between magnesium ions and bile

salts, a series of experiments was performed. When the magnesium sulfate solution, in four experiments, was placed outside of the collodion tube, despite intravascular injection of bile salts, no thrombi appeared in the collodion tube, or if they did, they were scarce and very small. It appears certain that magnesium ions went through the membrane into the circulation, and acted on the platelets and leucocytes in such a manner that the cellular thrombotic elements, passing down along the wall of the sac, were rendered less prone to agglutinate, and were carried away before they were precipitated, also the magnesium sulfate may act to destroy the platelets. When a solution of bile salts surrounded the collodion sac and magnesium sulfate solution was injected into the circulation, the formation of white thrombi was undoubtedly retarded to a certain degree, in regard to number and size, in spite of the stimulating action of bile salts.

This suggests that magnesium sulfate in small amounts will prevent thrombotic elements, especially platelets, from adhering to a pathologically changed vessel wall, partially owing to the platelet-destroying action of magnesium. This may serve to prevent thrombus formation. Possibly the phenomena may be ascribed to changes in surface tension.

CONCLUSIONS.

1. Distilled water dialyzes through the collodion tube and causes hemolysis. Clotting of the hemolyzed blood in the collodion tube occurs later.

2. Bile salts accelerate the appearance and development of white thrombi in the heparinized animal. The masses of white thrombi are very loose, soft, fragile and easily broken into clumps of platelets. They are poor in fibrin or the fibrin is abnormal, since they have less retractility than those obtained from the use of physiologic sodium chloride solution or calcium chloride or serum. Despite the increase in number and size of white thrombi, normal clotting does not occur.

3. When the collodion tube is surrounded by 1 or 2 per cent *d*-glucose solution, tiny and numerous white thrombi appear as radiating figures. The masses of white thrombi are rather loose and fragile. The clotting time of the heparinized blood does not appear to be shortened.

4. When a 1 to 2 per cent solution of calcium chloride is used as the dialyzing fluid outside the collodion tube, or when it is injected into the circulation, the formation of white thrombi is accelerated. They grow very rapidly. In spite of the action of heparin, the white thrombi formed are not so fragile as when bile salts are placed outside of the collodion tube. Fibrin seems to form easily. Obstruction of venous cannula takes place speedily and if the clots in the cannula are not removed, the white thrombi in the collodion tube remain small and become red by sedimentation of red cells.

5. Intravenous use of 10 per cent solution of magnesium sulfate without heparin retards the coagulation of circulating blood and permits the blood to flow through the extracorporeal loop from three to four times as long as normal. The formation of white thrombi, as well as red, is retarded. Magnesium sulfate (1 per cent) in physiologic sodium chloride solution placed outside the collodion tube markedly retards the formation of white and red thrombi in the heparinized animal. Magnesium sulfate (10 per cent), 50 mg. for each kilo of body weight each hour, administered intravascularly in the heparinized animal definitely prevents the first stages of thrombosis, and consequently prevents clotting.

6. It is possible by the combined use of adequate amounts of magnesium sulfate and of heparin intravenously to prevent all stages of thrombus formation for from 1 to 3 hours.

STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

VI. Certain Technical Modifications in Methods of Study.

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In the first paper of this series, a method for the study of extracorporeal thrombosis was presented by Rowntree and Shionoya. The technic outlined by them was followed closely by Shionoya in all of his subsequent experiments, as reported in Papers II, III, IV and V. Later the problem was approached from a somewhat different angle, and it was found desirable to modify the original technic. The discussion of such modifications and extensions forms the basis of this report.

Paraffin-Coated Apparatus.

In the original method a portion of the apparatus was coated with melted paraffin, but the cannulas and the rubber collars were left with clean uncoated surfaces. In control experiments with this technic the flow through the apparatus persisted only for from 6 to 10 minutes (rarely 25 minutes if animals were exceptionally large and vigorous), before occlusion of the lumen occurred. It was thought that complete coating of all parts of the apparatus except the collodion membrane might yield results of interest. To this end, the six glass sections and the four rubber collars were assembled and dipped into melted paraffin, before the collodion tube was attached. The paraffin was cooled carefully in order to obtain as smooth an inner lining as possible.

With such preparation of the apparatus, the flow of blood through the loop could be maintained with ease in normal animals for from 2

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to 5 hours instead of from 6 to 10 minutes, a striking demonstration of the important part played by physical variants in thrombosis.

The explanation of this tremendous increase in the duration of flow was not far to seek. With the original technic the glass side tubes were coated with paraffin and then inserted into the uncoated rubber collars until the ends met. If it were possible to secure a water-tight joint by this means such a procedure would be as effective as the coating of the apparatus with paraffin in the assembled state. However, this was not possible, since the ends of the tubes were not ground to fit closely. Thus, when blood passed from one paraffined tube into the next a portion of it came in contact with the surrounding rubber collar and, this being an unparaffined ("water-wettable") surface it facilitated the attachment and disintegration of platelets with the consequent formation of a thrombus at this point. Furthermore,

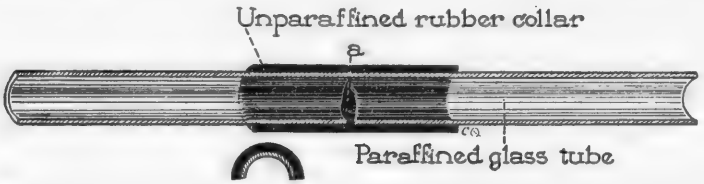

FIG. 1. Sagittal section through joint of extracorporeal loop (early technic). *a*, point at which blood came into contact with unparaffined rubber collar.

since at this juncture the lumen of the apparatus expanded suddenly, eddies were formed, thus fulfilling another prerequisite for the formation of thrombi (Fig. 1). When one considers that there were four such points in the original apparatus and that the surfaces of the two cannulas also were clean and unparaffined, it is not difficult to understand how thrombi could obstruct the lumen in one of these areas within 10 minutes in the normal animal.

If one is studying the duration of flow with and without anticoagulant substances (as did Shionoya) the original technic is far better than the modification since it yields more contrast in results and permits the comparative study of duration of flow through the extracorporeal loop. If, on the other hand, one wished to follow, microscopically, the process of thrombosis, the newer method is

preferred since it allows adequate time for detailed study of the early stages of thrombosis.

Size of Collodion Tube.

The collodion tubes used in the first method were 7.5 mm. in diameter and the inside diameter of the glass tubes was but 2.5 mm. Thus, the lumen was suddenly expanded to three times its previous size as the blood left the arterial corner tube and entered the collodion tube. The formation of eddies was marked and the speed of the stream was profoundly decreased. As a result the deposition of platelets and the formation of obstructing thrombi were correspondingly hastened. The large membranes, too, assumed wrinkles of considerable size at points of attachment, another factor predisposing to early thrombosis.

In a later study, tubes just large enough to slip over the ends of the glass tubes were utilized. The pulsation in these is less marked, a factor worthy of attention in microscopic observations, and there is less tendency to early obstruction since the blood current is more rapid and forcible when the diameter of the lumen is decreased.

Direct Microscopic Observation of Thrombus Formation.

A microscope and a carbon arc lamp have been added to the apparatus, making it possible to follow the course of events in the transparent collodion tube throughout the experiment. Using the low power objective as a water immersion lens, the surface flow of blood in the tube can be distinctly seen as well as the gradual formation of white thrombi from their earliest stages. Vital stains have been utilized in this connection. 1 per cent methylene blue introduced into the circulation is taken up by the platelet thrombi, and thus they are outlined clearly against the prevailing red background.

Rapid Method of Permanent Staining of Thrombi on the Collodion Membrane.

As was stated in Paper I, the membranes may be "observed directly, or studied under the microscope with or without the aid of special stains; or the specimen may be prepared and embedded in paraffin for

section staining and ordinary pathologic study." The latter method was used as a routine since it yielded permanent specimens. It possessed, however, outstanding disadvantages; the time element was considerable as the sections went through the busy routine of the pathologic section, and unless there was a considerable mass of thrombi on the membrane the vertical section would show practically nothing. For a study of the early stages of the formation of thrombi on the membrane, this method was wholly inadequate.

To supplement the direct observations through the low power of the microscope, it was desirable to study the membranes with counter-stains, and the higher magnifications. It was found difficult and unsatisfactory to do this while the membrane was attached to the apparatus. Attempts were then made to stain the tubes immediately following removal from the loop. After considerable experimentation, a satisfactory method was devised for staining and observing the minute details of early thrombosis on the surface of the membrane. The tube is washed gently in 0.9 per cent sodium chloride solution immediately after its removal from the apparatus and segments about 1 cm. square are clipped out. Some of these small portions are fixed in the vapor of 1 per cent osmic acid. A few drops of dilute ether-alcohol-collodion solution are placed on a clean glass slide and the membrane specimen dried quickly on the outer surface with a blotter or soft cloth. Then before the collodion solution has dried the membrane is placed on it (with the thromboplastic elements uppermost) and gently flattened. The preparation is allowed to dry for a few seconds and is then placed in distilled water for from 2 to 3 minutes. If the membrane does not wash off in the water, it probably will adhere to the slide during the staining process. The slide is then run through a hematoxylin-eosin-alcohol series and finally through carboxylene and xylene exactly as though it were a paraffin section. It is mounted with Canada balsam under a cover slip. The entire process occupies only from 15 to 20 minutes, and several sections may be prepared at the same time.

With this method membranes can be removed from the extracorporeal loop after but a few seconds or minutes of flow and the individual platelets which are attached can be studied long before a tendency toward grouping is manifest. Serial sections are also possible and

thus the gradual growth of a thrombus from the first tiny clump of platelets to the leucocyte, fibrin and red cell stage may be followed and preserved in permanent sections. These sections are really bird's-eye pictures of the progress of events on the surface of the membrane, and supplement adequately the observations made directly with the arc lamp and low power objective during the course of the experiment.

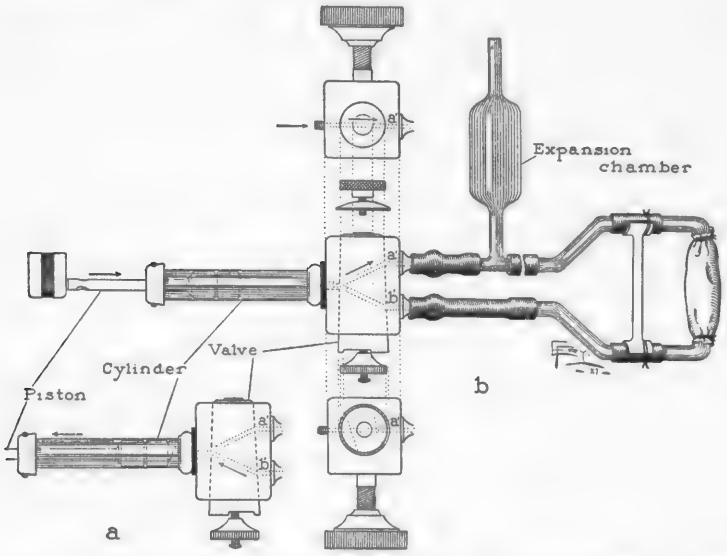

FIG. 2. *a*, piston moving outward (opposite *b*) and current through *b'*, while *a'* is closed by valve. *b*, method of attachment of extracorporeal loop to intake and discharge pipes (*a'* and *b'*) of Woodyatt pump. Piston moving into cylinder—current through *a'* while *b'* is closed by valve.

Artificial Heart.

The extracorporeal loop, minus the cannulas but supplied with an expansion chamber, was attached to the two valves of the Woodyatt pump. All surfaces were carefully coated with paraffin so that at no point would the contained blood come into contact with a water-wettable surface. Whole blood was run directly into the apparatus from a greased cannula in the carotid artery of a rabbit. The motor

was started immediately and a continuous flow of blood through the loop was obtained. Thrombus formation in the collodion tube proceeded at the same rate as in the normal animal, but the thrombi were laid down in longitudinal streaks because of the absence of eddies.

The alternate suction and expulsion of the single cylinder of the pump imparted an intermittent motion (stop-flow-stop-flow) to the blood in the apparatus. The expansion chamber overcame this disadvantage to a certain extent and a fairly continuous flow was finally obtained. Eddy formation being minimal because of the slow speed of the stream, white thrombi (and later, fibrin) were laid down in longitudinal striations, instead of in the usual manner. This is another confirmation of the importance of eddy formation in the growth of thrombi (Fig. 2).

Platelet-containing oxalate plasma was used in the same manner; on recalcification some platelet deposit and fibrin formation was evident. Since the staining method had not been worked out at this time, no microscopic studies were possible.

Additional Modifications.

Instead of the test-tube dialyzing jacket originally used, a small chamber 2.5 by 7.5 by 1.7 cm. made of glass cemented with balsam has been substituted. This chamber is more stable, holds more fluid and permits agitation of contents while dialyzing experiments are carried on. It is also better adapted for use when direct microscopic observations are being made.

In the selection of arterial cannulas, it is well to use the largest one which can be slipped into the vessel. As has been shown elsewhere, the more the cannula is constricted the more easily do obstructing thrombi form.

The medial branch of the external jugular vein of the rabbit possesses a valve system near the point of juncture which prevents reflux of blood into this branch. Consequently when the vein is ligated above this point it promptly collapses and it cannot be refilled except from above. It may be incised and a cannula inserted, without the necessity of clamping, since there is no loss of blood. Thus unnecessary trauma to the delicate intima is averted and the formation of thrombi in the vein practically ceases to be a consideration.

CONCLUSIONS.

As the result of these modifications, many of which in themselves may appear trifling, the course of events in the extracorporeal loop is greatly affected. White thrombi, it is true, are laid down in from 10 to 20 minutes, but the formation of fibrin sufficient to cause obstruction is prevented for 3 hours or more. Thus, the observations in normal animals tend to approach those described in Paper III, after the use of anticoagulants such as heparin and hirudine. The new technic offers certain advantages in the study of the details of the early stages of thrombosis.

THE MECHANISM OF THE INFLAMMATORY PROCESS.*

I. The Electrophoresis of the Blood Cells of the Horse and Its Relation to Leucocyte Emigration.

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The present status of the relationship between the electrokinetic potential of leucocytes and the potential differences existing between injured and uninjured tissue zones, points to a situation which is not alone unsolved quantitatively but also qualitatively; but its solution presents, perhaps, a hopeful answer to the question of leucocytic migration in response to injury.

HISTORICAL.

Dineur (1) (1893) seems to have been the first investigator who attempted to study the electrical charge on white blood cells. He placed platinum wires that had been sealed into glass capillaries into the normal and inflamed peritoneal cavities of the frog. Leucocytes tended to enter the capillaries in the absence of a current. In normal frogs, upon the passage of a current, more leucocytes went to the anodal capillary, but just the reverse occurred in frogs with peritoneal exudates. In the latter, the leucocytes seemed to be positively charged, wandering in greater numbers to the cathode. From that time until the present no work has apparently been done which defines *quantitatively* the speed of leucocytic migration and a possible ζ potential. Further, the conditions of the previously undertaken investigations have been so ill defined according to our present knowledge of the behavior of charged colloidal particles, that the investigators' statements seemingly disagree even as to whether leucocytes wander to the anode or to the cathode.

Lillie (2) (1903) studied the white blood cells of the frog suspended in isotonic

* Unless otherwise stated in this and the following papers, the word "inflammation" is used in a strictly limited sense; *i.e.*, the wandering of a white blood cell to a point of injury.

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sugar-NaCl solution. Under these conditions it was found that lymphocytes wandered to the anode; the polymorphonuclear leucocytes to the anode or cathode, or even remained stationary. Höber (3) the following year maintained that frog leucocytes were negatively charged. In 1914, Schwyzer (4), overlooking the definite results of Lillie and Höber, stated that the work of Dineur was untrustworthy, and that his own efforts to produce cataphoresis of leucocytes in tissues were unsuccessful. Schwyzer, however, was the first to hint that electrical differences of potential may play a part in producing the appearances of inflammation. He states: "Der Weg zur Emigration wird durch die Elektronenströme angedeutet, und die Pseudopodien werden durch diese Ströme in der schwachsten Stelle der Membran [capillary wall] gerichtet. Die Leucocytenwanderung im Gewebe wird also *chemotaktisch* aufgefasst, konnte aber auch durch Ionenwirkung erklärt werden." Mendelssohn (5) observed that leucocytes wandered to the cathode. The work done by Höber and his coworkers later leads him to state (6): "Dies hängt damit zusammen, dass nach der Kataphoresegeschwindigkeit und nach der Entladbarkeit mit La^{+++} beurteilt, das ζ potential der Leucocyten nur schwach, negativ, null, oder schwach positiv ist." Eliasoph (7) found that gelatin suspensions of leucocytes wandered to the platinum anode, and that if the electrodes were buried in splenic tissue, there was also anodal migration. Unfortunately, non-polarizable electrodes were not used by Eliasoph. Feringa (8) (1922, 1924) mentions qualitative experiments with rabbit and other white cells. He found them to be negatively charged, but no quantitative data are given concerning speed or potential. Feringa states that lymphocytes do not move as quickly as leucocytes cataphoretically. This will subsequently be shown to be probably a misinterpretation. This author, agreeing with Schwyzer, believed that the potential difference between injured and uninjured tissues caused leucocytic migration to a point of injury, the basis of this taken as pH changes. His observations that leucocytes in general move with ameboid movements toward the anode on an agar floor had not been noted by Abramson (9) on a glass floor for a small potential difference. However, studies on the *floor* of an electrophoresis chamber are complicated by at least the following: (1) the nature of the floor and its influence on the particle itself; and (2) the fact that the suspending medium is also charged relative to the floor. In general, alkaline fluids near the floor of a cataphoresis chamber are positively charged and move toward the cathode. Before one can state, therefore, that ameboid motion is influenced by an electric current, it must be ascertained whether leucocytes would migrate against a moving stream of fluid as occurs in the instance of spermatozoa which move *against* the ciliary stream in the ducts of the female generative organs.

Abramson (9, 10) during the same year (1924) published studies on human lymphocytes suspended in serum. He found human lymphocytes to be definitely negatively charged and estimated the mean

cataphoretic velocity (C.V.) semiquantitatively at 0.3μ per volt per cm. per sec. Further, lymphocytes kept on ice during a period of 30 hours did not appreciably alter the speed of migration. The work on human lymphocytes was undertaken in order to test a hypothesis then proposed which attempted to correlate *quantitatively* the order of magnitude of the potential differences in injured tissues and the speed of white cell cataphoresis (see Fig. 2). The researches of Du Bois-Reymond (11), Biedermann (12), and Hermann (13) pointed to the fact that negative electricity could be conducted from injured tissue surfaces. Potential differences between injured and uninjured surfaces up to about .1 volt had been recorded. With this in mind, it seemed possible that the border of a zone of injury relative to the comparatively uninjured cells, capillary wall, and blood stream could be positively charged. As the surrounding tissue and tissue fluids are conductors, a continuous current must flow between uninjured and injured tissues, the energy coming from the metabolic changes incidental to injury. It was then shown that the order of magnitude of the potential drop between uninjured and injured tissues was probably the same as that necessary to cause white blood cell migration as far as lymphocytes were concerned. That this is also true for polymorphonuclear leucocytes as well will be shown in this communication, and data will be presented concerning the absolute velocities of horse polymorphonuclear leucocytes and lymphocytes in plasma and serum. Observations also have been made concerning cataphoresis in gels and their viscosity. The significance of these data in relation to the inflammatory process will be demonstrated.

Methods.

The method in detail of determination of the C. V. of cell migration is presented elsewhere in connection with the more physical aspects of the present studies (14). In brief, a microscopic method, similar to that used by Northrop (15), was employed which permitted fairly accurate measurement of red cell velocity at different levels in the cataphoresis chamber. From these data the velocity of the medium, V_m, in a given level of the cell could be calculated. The actual velocity of the white cells, V, is then,

$$V = V_o - V_m$$

where V_o is the observed velocity in the particular level. The potential drop in the cell during the measurements was usually of the order of 15 volts per cm.

The Calculation of ζ Potential and the Viscosity of the Suspending Medium.

As is well known (16) the flow of certain colloidal solutions through a capillary, in particular the lyophilic sols, does not follow the Poiseuille formula. That is, with different pressures exerted on the moving column, different values of "viscosity" are obtained. The smaller

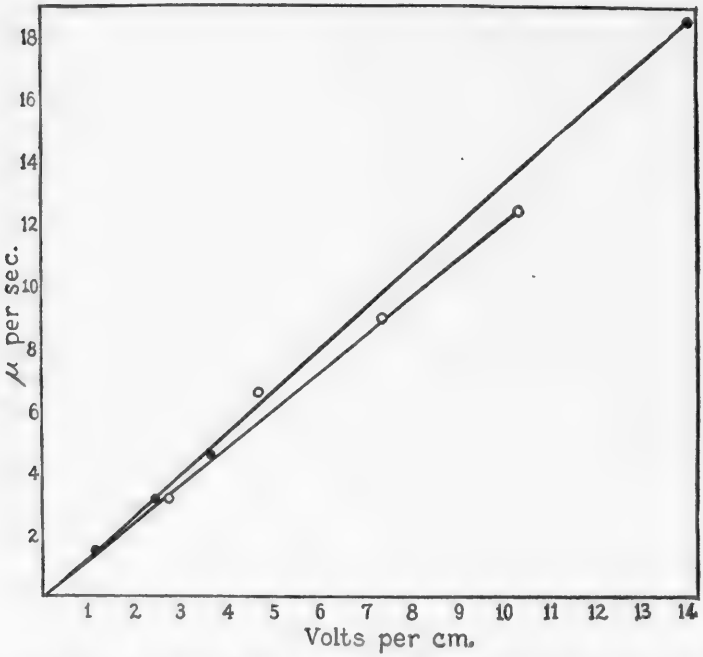

FIG. 1. The C. V. of red cells in plasma is proportional to the applied P. D. The same linear relationship exists for serum.

the pressure, the greater is the "elasticity" of the solution. Blood shows this elastic effect to a slight degree.

From consideration of the Helmholtz-Lamb equation, $v = \frac{HK\zeta}{4\pi\eta}$ (v = velocity, K = dielectric constant of the medium, H = potential drop per cm., ζ = electrokinetic potential, η = viscosity)

(all units are electrostatic), it follows that in a system where the elasticity of the medium could hinder migration, v would not be proportional to H.

Fig. 1 shows that marked differences in H produce a particle velocity which follows the conventional Helmholtz-Lamb equation, and consequently demonstrates that under these conditions plasma and serum have no "elasticity." Accordingly taking the value of .02 for η,[1] 85 for K (Fürth), and the observed values for the other factors, it follows that for a drop in potential of 1 volt per cm. at 18° the electrokinetic potential

$$= \frac{4 \times 3.14 \times .02 \times (300)^2}{85}$$

$$= (26.5 \times \text{velocity in } \mu \text{ per sec.}) \text{ millivolts.}$$

Evidence has also been obtained by Freundlich and Abramson (14) that the migration of the micellæ of certain "elastic" gelatin gels has the same speed in the gel that is found in the sol. In this instance, a linear relationship between H and C.V. has also been found. This strongly suggests that extremely small differences of potential existing in tissues could produce particle migration even though a viscous gel-like medium was present.

The Electrophoresis of Erythrocytes.

Plasma suspensions of leucocytes and red cells were obtained by oxalating 1 litre of freshly shed horse blood with 8.5 cc. of the saturated solution of the potassium salts. Table I gives the C.V. of red cells in plasma obtained from seven different horses. The mean value .98 μ per volt per cm. per sec. for plasma agrees quite well with the mean value, 1.01μ per volt per cm. per sec. obtained in serum from ten other horses. The data for serum[2] are published elsewhere (14).

[1] The viscosity of serum is actually 5–10 per cent lower than that of plasma. This difference is here neglected.

[2] The data for serum include studies made on aggregates of red cells and red cell rouleaux. It was found that the C. V. of different shaped aggregates were identical with one another and with single cells. The significance of these facts is discussed (14).

Keeping the cells on ice during a period of 3 days or less did not change the C.V. It may be recalled that the isoelectric point of fibrinogen is in the region pH 8.0. Plasma contains .1 to .4 per cent of this protein. The isoelectric point of the other proteins present is in the region pH 5.0. As the isoelectric points of the proteins influence the ζ potential, different C.V. could have been expected from plasma and serum cell suspensions.

TABLE I.

The Electrophoresis of Red Cells in Plasma. Seven Different Horses.

Plasma	Age of cells	Speed	ζ potential
	hrs.	*μ per sec. per volt per cm.*	*millivolts*
A	30	1.04	27.5
B	30	1.02	27.0
B		.90	24.0
C	6	1.07	28.0
C serum	6	1.06	28.0
C	30	1.01	27.0
F	6	.97	26.0
F serum	6	.94	25.0
H	6	.91	24.0
I	6	.90	24.0
I		1.01	27.0
J	6	.95	25.0
Mean plasma		.98	26.0
Mean serum (10 horses)		1.01	27.0

The Electrophoresis of White Cells.[3]

Polymorphonuclear leucocytes (P) wander in plasma (Table II) with a mean C.V. of .54μ per volt per cm. per sec. (seven horses). This value is about half that found for red cells from the same group

[3] The C. V. of blood platelets has also been studied and will be presented in a subsequent communication.

(98:54). The calculated mean ζ potential is 14.5 millivolts. Small lymphocytes (SRC) wander in plasma about 20 per cent faster than polymorphonuclear cells. Because of the presence of blood platelets, optimal optical conditions were not present for differentiation of polynuclear cells and round cells. For this reason, serum was studied in greater detail; but it was quite clear that the same relationships held for serum and plasma. In both instances, as found previously for human lymphocytes, keeping the cells on ice up to 48 hours did not alter the C.V.

TABLE II.

The Electrophoresis of White Blood Cells in Plasma (Seven Horses).

(P) = polymorphonuclear leucocyte. (SRC) = small lymphocyte.

Plasma	Age of cells	Corrected speed	ζ potential
	hrs.	*μ per volt per cm. per sec.*	*millivolts*
1 (P)	30	.51	13.5
1 (SRC)		.61	16.0
2 (P)	6	.46	12.0
3 (P)	6	.59	15.5
3 (P) serum	6	.50	13.5
3 (P)	30	.57	15.0
4 (P)	6	.57	15.0
5 (P)	6	.47	12.0
5 (SRC)	6	.60	15.5
6 (P)	6	.58	15.5
7 (P)	6	.58	15.5
Mean (P).....		.54	14.5

$$\frac{\text{Red cell}}{\text{P}} = \frac{98}{54} = 1.8$$

Table III is a summary of experiments with cells in serum taken from six different horses; the mean velocity of polymorphonuclear leucocytes observed in this series of experiments was .51μ per volt per cm. per sec. The calculated ζ potential was 13.5 millivolts. Lymphocytes have given a mean velocity of .60μ per volt per cm. per sec. with a ζ potential of 17.0 millivolts. It is preferred to take the values given in the tables for large round cells (LRC) as approximate because of the difficulties incidental to differentiation of unstained cells.

TABLE III.

The Electrophoresis of White Blood Cells in Serum. Six Different Horses.

(LRC) = large round cell.

Type of cells	Age of cells	Corrected mean (V)	ζ potential	Remarks
		μ per volt per cm. per sec.	*millivolts*	
1 P	30 hrs.	.50	13.0	P is a mean of 3 values .49, .58, .43 obtained from *different* levels in cell. These do not agree as well with one another as those given below taken in the midregion.
SRC		.59	15.5	
2 P	6 "	.51	13.5	Although a different chamber than above was used in this experiment, the agreement is excellent P — SRC are mean of 2 experiments.
SRC		.60	17.0	
2a P	30 "	.50	13.0	
SRC		.62	16.5	
LRC		.55	14.5	
3 P	3 days	.61	16.0	In this serum, 3 days old, although higher values have been noted, the ratio P/SRC is practically the same as above.
SRC		.74	14.5	
4 P	2 "	.55	14.5	
SRC		.66	17.5	
5 P	6 hrs.	.47	12.5	
SRC		.54	14.0	
6 P	6 "	.54	14.5	
Mean as above: 1 P, 2 P, 2a P, 4 P, 5 P, 6 P		.51	13.5	$\frac{\text{SRC}}{\text{P}} = \frac{17.0}{13.5} = 1.25$
1 SRC, 2 SRC, 2a SRC, 4 SRC, 5 SRC		.60	17.0	$\frac{\text{Red cells}}{\text{P}} = \frac{27}{13.5} = 2.0$

TABLE IV.

Typical Protocols of Experiments to Determine Leucocyte Velocity. Horse Serum and Cells.

Column 1, 6 hrs. old. Column 2, 30 hrs. old.

No.	Type	V_0	V	ζ potential		No.	Type	V_0	V	ζ potential	
				millivolts						*millivolts*	
1	P	.73	.48	12.5		1	P	.76	.54	14.0	
2	P	.68	.45	12.0		2	P	.70	.48	12.5	
3	SRC	.87	.62	16.5	Chamber refilled	3	P	.72	.50	13.5	
						4	P	.69	.45	12.0	
4	P	.71	.48	12.5		5	SRC	.82	.60	16.0	
5	P	.75	.55	15.0		6	LRC	.79	.55	14.5	
6	P	.77	.57	15.0		7	LRC	.81	.60	16.0	
7	P	.77	.57	15.0		8	SRC	.90	.67	18.0	
8	P	.68	.47	12.5		9	SRC	.76	.56	15.0	
9	P	.71	.51	13.5		10	SRC	.97	.72	19.0	
10	SRC	.85	.64	17.0		11	LRC	.76	.51	13.5	
11	LRC	.65	.44	11.5		12	SRC	.82	.61	16.0	
12	SRC	1.01	.81	21.5		13	SRC	.90	.65	17.5	
13	P	.69	.49	13.0							Refilled
14	P	.74	.53	14.0		14	P	.63	.47	12.5	
15	P	.67	.46	12.0		15	P	.68	.41	12.5	
16	P	.93	.73	19.0		16	SRC	.76	.61	16.0	
					Refilled	17	P	.79	.46	12.0	
17	SRC	.79	.59	15.5		18	P	.74	.52	14.0	
18	P	.76	.54	14.5		19	P	74	.52	14.0	
19	P	.68	.48	13.0		20	P	.74	.52	14.0	
20	P	.76	.57	15.0		21	P	.69	.47	12.5	
21	P	.75	.54	14.5		22	SRC	.79	.54	14.5	
22	SRC	.83	.62	16.5		23	SRC	.86	.61	16.0	
23	P	.70	.49	13.0		24	P	.79	.55	14.5	
24	SRC	.90	.69	18.5		25	LRC	.79	.57	15.0	
25	LRC	.90	.66	17.5		26	SRC	.84	.63	16.5	
26						27	P	.69	.45	12.0	
						28	SRC	.83	.60	16.0	
						29	P	.76	.53	14.0	
						30	P	.76	.53	14.0	
Mean P.....			.51	13.5		Mean P.....			.50	13.0	
Mean SRC.....			.66	17.5		Mean SRC.....			.67	16.5	
						Mean LRC.....			.55	14.5	

The C.V. of single polynuclear cells in serum and plasma was quite constant. In the same preparation (Table IV, Column 1) all but one cell of this type wandered with a speed which varied between .45 and .57μ per volt per cm. per sec. This variation appears to be much less from *qualitative* observation. Three or four leucocytes accidentally suspended at the same level migrate with the same velocity. The mean value, therefore, of the sixteen cells closely approaches the true value of the absolute velocity for the preparation, .51μ per volt per cm. per sec. The mean ζ potential, calculated as described heretofore is (26.5 $\times$ .51) 13.5 millivolts.

The behavior of the lymphocytes is as interesting as it is inexplicable. Although a smaller variation in velocity was expected because of the regularly spherical shape, as the table indicates, the velocity of these round cells varies considerably. In general, it may be stated the small lymphocytes wander about 15 to 30 per cent faster than polymorphonuclear cells. The six lymphocytes whose velocities are noted had a mean speed of .66μ per volt per cm. per sec. The calculated potential is (26.5 $\times$ .66) 17.5 millivolts. Frequently, round cells are observed which migrate more slowly than polymorphonuclear leucocytes. Cell 11, for example, was of the large mononuclear type (LRC). Its migration speed was only .44μ per volt per cm. per sec. On the other hand, Cell 6, a polymorphonuclear leucocyte, migrated with a velocity of .73 per sec. These differences are all the more striking when observed. The lymphocytes overtake the leucocytes; the red cells go swiftly by with a velocity practically twice as fast; and then suddenly, one may observe a round cell overtaking another round cell, or a leucocyte exhibiting the unusual property of migrating faster than a lymphocyte. This, however, is rare. On the other hand, although potential drops per cm. as high as 30 volts had been used, leucocytes (20° C.) that had settled to the glass exhibited no movement in either direction and could not be removed by a powerful stream of saline forced through the electrophoresis chamber. Feringa's experiments with an agar floor were not repeated because of the questionable influence of water flow on the ameboid movements of the cells mentioned previously. The data, however, given in the tables represent *absolute* velocity within the cells irrespective of the water flow and of ameboid movements.

DISCUSSION.

It has been stated that the present study was a continuation of the work previously done on human lymphocytes in an attempt to correlate *quantitatively* the electrokinetic potential of white blood cells with the differences of potential passing between injured and uninjured tissues (9, 10).

The following discussion is in continuation of this attempt to establish a quantitative basis for this theory of the mechanism of inflammation. It may be again emphasized here that other views, such as those of chemotaxis, of surface tension, etc., are not meant to be excluded. As will be shown, however, there are two quantities: (1) the speed of white cells per volt per cm., per sec.; and (2) the P.D. existing in living injured tissues, whose order of magnitude can be presented with a fair degree of certainty—a degree of certainty which will permit of a correlation of the two, and of a definition in a limited sense of several physicochemical factors incidental to the inflammatory process.

The preceding data lead one to suspect that mammalian white cells are negatively charged and move with an appreciable velocity under a P.D. of 1 volt per cm. toward the anode. With this in mind, the nature and order of magnitude of the P.D. between injured and normal tissues will be described. It is well known that the *surfaces* of injured tissues are negative to the uninjured surface (15). That the uninjured surface of an injured muscle is comparable to the uninjured underlying tissue is shown by the following experiment. If a carefully dissected frog muscle be cross-sectioned, the injured surface at the moment after cutting shows only a small fraction of the maximum E.M.F. which appears only after a considerable interval (17, 11, 13).

Differences in potential arising in tissues incidental to injury cannot have their sources in electrode potentials as no metal is present. Consequently the currents passing between injured and uninjured tissues must have their origins either in: (1) diffusion potentials which are based upon differences in ionic molarities, or (2) membrane potentials, potentials produced by intervening phases—most probably both. From the former, slight electromotive forces are to be expected not greater than .01 volt. On the other hand, membrane potentials are

usually of the order of .02 to .1 volt. Michaelis' discussion of the physiological significance of diffusion potentials does not give them a particularly important position (18). In the instance of an injured tissue zone, the presence of a new phase would, it is true, produce in all

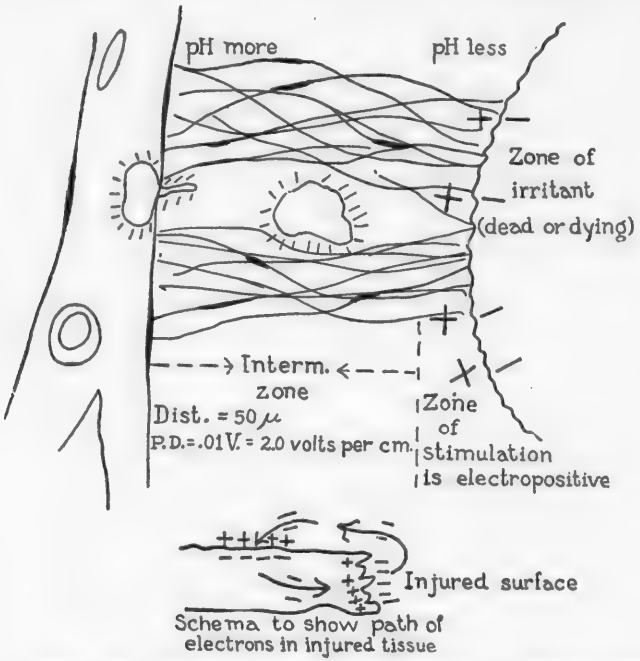

FIG. 2. Note the absence of intervening membranes in the spaces where leucocytic migration occurs between injured area and capillary wall. This *structural* homogeneity lends aid to the supposition that the conductivity and hence the drop in potential is fairly uniform. For further description see text.

probability potential differences of the order of magnitude possessed by membrane potentials.[4] The main drop in potential, however, may be across the phase producing the potential. A particle, migrating

[4] As for example in muscle. A resting muscle is isoelectric. With the production of an injured surface the varying permeability of the membrane of the muscle cells for anions and cations may produce P. D. of the order of magnitude of membrane potentials.

cataphoretically, would thus be out of range of the main drop in potential except when migrating through the phase—*e.g.*, the wall of a capillary. That diffusion potentials may play a significant rôle in the production of cataphoretic migration is shown by the following.

Reference to Fig. 2 shows that negative electricity is drawn from an injured surface. Consequently electrons flow from uninjured tissue zones to injured tissue zones, making the injured area electropositive. That an injured area is relatively positive is made all the more probable by the fact that injured tissues have a lower pH than the blood in the

TABLE V.

This table demonstrates that even with minimum potential differences between injured and uninjured zones appreciable drops in potential per cm. should be found, even with maximum distances.

Distance between injured zone and capillary or normal tissue.....	100 μ	50 μ	25 μ	10 μ
Possible potential differences between injured and uninjured tissue	Drop in potential in tissue			
volts	*volts per cm.*	*volts per cm.*	*volts per cm.*	*volts per cm.*
.001	.1	.2	.4	1.0
.005	.5	1.0	2.0	5.0
.010	1.0	2.0	4.0	10.0
.050	5.0	10.0	8.0	20.0
.100	10.0	20.0	40.0	100.0

capillaries and adjoining normal tissues (19). Consequently, the higher hydrogen ion concentration (see Fig. 2) again would lead one to believe that injured tissue was electropositive.

Before discussing the order of magnitude of the P.D. existing between injured and uninjured zones, it should be recalled that P.D. is expressed in *volts per cm.* A picture of the forces at play which may influence a charged leucocyte is appreciated only if the drop in potential is converted by estimation of the distance from the injured point to the nearest capillary, to P.D. per cm. Table V shows the estimated order of magnitude of the P.D. per cm. with different distances and different potential differences. The fall across the injured tissue to capillary area would have to be divided inversely proportionally to the resistance of the tissues. As relatively normal tissues have a high resistance

and as the tissue fluids similarly are not good conductors, it is fair to assume that even though the total drop in potential is not uniform, a portion of it, sufficiently great to influence migration of a charged particle, is present. It should be noted that *the inflammatory process takes place in connective tissue*, and leucocytic migration occurs in the capillary spaces between fibrous tissue bands where the P.D. is unhindered by the presence of cell membranes existing in muscle. From the point of view of the structure of the intracellular fluids in connective tissue, one may assume for the present that the thinness and conductivity of phases or membranes present do not make the curve of potential drop sufficiently unlinear to reduce the probability of the correctness of the order of magnitudes to be considered.

Table V shows that even with a P.D. of .001 volt between injured zone and a capillary wall .1 mm. away, the P.D. per cm. is .1 volt. Of course, this is an extreme instance. The potentials measured between cut surface and uninjured surface vary between .030 and .100 volt, and if membranes (phases) are present in the injured zone, this order of magnitude of potential would be expected. If, however, only diffusion potentials incidental to the enormous rise of metabolism which occurs (20) are present and if the P.D. in that instance be only 5 millivolts, with a distance of .05 mm., the P.D. per cm. is *1 volt*,—a force sufficient to bring a leucocyte moving in plasma at the rate of .5μ per volt per cm. per sec. to the point of injury in 2 minutes.

Why do not the red cells and lymphocytes also migrate at once toward the point of injury? Some of them do, but the great difference between these cells and polymorphonuclear leucocytes is that the ameboid cells stick to the capillary walls and are uninfluenced by the rush of blood going by (see Paper II of this series). The speed of blood in capillaries is remarkably constant throughout the vertebrate kingdom. One may expect in mammals a speed of about .5 mm. per second of the cells passing through certain capillaries (21). Even if the P.D. per cm. between blood and injured zone were 20 volts, the speed of a cell moving toward the zone of injury at a rate of .5μ per volt per cm. per sec. would be only 10μ per sec. Thus, there would be a force exerted on the blood cell in the direction of the blood stream fifty times as great as that exerted in the direction of the point of injury (Fig. 2). When, however, a white cell is trapped by the capillary wall, the electromotive

or other forces are dominant and the forward pressure of the blood stream on the adherent cell is negligible. This presupposed, of course, that leucocytes are able to wander through the wall, as they can. The same phenomena are readily seen in an electrophoresis chamber of the kind described above. At *room* temperature, on the floor of the cell, are stationary polymorphonuclear leucocytes, and hovering about apparently suspended in the immediate fluid strata are lymphocytes and red cells. Even though the P.D. on occasions has been 30 volts per cm., the leucocytes which have stuck to the glass remain there uninfluenced by the difference of potential while the round cells and red cells move swiftly by just as in a blood vessel.

One further point at present remains to be discussed—the electrophoresis of water. Granting the difference of potential and the other factors that have been previously discussed, it follows that the movement of the tissue fluids would also be influenced. As the experiments of Mudd (22) have conclusively shown, animal membranes are negatively charged when the medium is serum or any other alkaline fluid. The significance of this in relation to the inflammatory process is at once evident. There should be adjacent to the strands of connective tissue and in the pores of the connective tissue a cathodal flow of water. But, these strands of connective tissue form capillary spaces. If these spaces are closed systems, as in an electrophoresis chamber, in the absence of structural irregularities, there may be in the midregions of these capillary spaces a return anodal flow to assist leucocyte migration where the capillary spaces are not too small.

SUMMARY.

1. The velocity of cataphoretic migration of blood cells in plasma and serum is proportional to the potential drop applied.

2. The cataphoretic velocity of red cells, polymorphonuclear leucocytes, small lymphocytes, and large lymphocytes is described for serum and plasma.

3. The relation between the electrokinetic potential of white blood cells and the differences of potential probably existing in injured tissues are correlated quantitatively.

4. This correlation suggests that migration of leucocytes to a point of injury is, in part, dependent upon the electromotive forces at play in the tissues.

The writer wishes to express his thanks to Professor H. Freundlich for his constant guidance and criticisms, and to Dr. Ettisch for his unceasing interest.

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THE MECHANISM OF THE INFLAMMATORY PROCESS.*

II. Concerning the Adhesive Force of One Pseudopod of a Frog Leucocyte and Its Relation to Leucocyte Emigration.

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Although much work has been done concerning the adhesive qualities of white blood cells, these studies of stickiness have apparently all been made *in vitro* with glass or other surfaces (1).

This communication deals with observations made on the behavior of a single leucocyte in a capillary of the web of a frog's hind limb. The phenomenon seems not to have been reported. Because of the quantitative considerations which may be deduced from this observation, it is worthy of description in detail. Dr. G. Ettisch was present and confirmed the sequence of events to be described in the following.

1 hour after the frog (*Rana temporaria*) had been anesthetized by urethane, the vascular changes incidental to an inflammatory process produced by a needle-HCl puncture of the web were studied. The vascular channels were wide open with a very rapid flow of blood cells, unhindered except at points where white cells had begun to stick to the capillary walls or roll slowly along the wall. At the bend of a capillary whose width was about four times the diameter of a polymorphonuclear leucocyte, an ameboid cell was seen to be suspended in a rushing blood stream by a single thin white thread of protoplasm (Fig. 1, *a*). The cell was in the centre of the stream, in contradistinction to the other cells along the wall, and was held in place by this thin thread of cytoplasm (about three times as long as the cell itself) which was drawn out to a minute point parallel to the direction of the blood

* See Paper I of this series.

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stream, and was stuck to the wall by an apex whose diameter seemed to be about 1 μ. The preparation as a whole was in the horizontal plane. The cell was definitely seen to be in the middle of the stream surrounded and tossed about by red cells streaming past so rapidly that individual cells could not be observed. The thin thread of cytoplasm (pseudopod) then shortened until the cell approached the capillary wall to about its own length (Fig. 1, *b*).

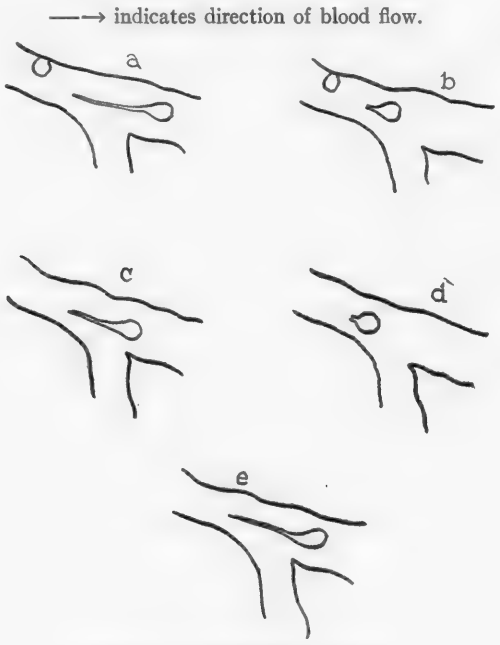

FIG. 1. For description, see text.

Then, seemingly overwhelmed by the force of the blood stream, the pseudopod was again stretched out to its previous length. After several minutes of such play, the pseudopod slowly contracted (Fig. 1, *c*) and the white cell was drawn to the capillary wall (Fig. 1, *d*). This lasted but half a minute when the cell was again drawn out by the force of the blood stream with a thread of cytoplasm as long and as thin as heretofore described (Fig. 1, *e*). After a short period of

tossing about in the centre of the stream, the pseudopod was apparently torn free from the wall and the cell disappeared from the field.

It is evident that the adhesive force exerted by this single pseudopod was equal at least to the resultant of the forces of the stream of blood on the white cell and of the force of gravity. To obtain an idea of the order of magnitude of this force, let it be assumed that the leucocyte was a sphere of radius, r, in a medium whose viscosity, η, was constant and whose speed, v, was also constant. Then, F, the force in dynes, is expressed by the following equation:

$$F = 6 \pi r \eta v$$

Substituting .03 for η, 4×10^{-4} for v, and .05 for v (2), the value of 10^{-5} dynes is obtained for the horizontal component, the force of the blood stream. As the cell was maintained by the blood stream in a horizontal plane, it is evident that the gravitational component was comparatively small. This vertical component is expressed by

$$F = \frac{4}{3} \pi r^3 (D\text{-}\delta) g$$

where r is the radius of the cell, D the density of the medium, δ the density of the particle, and g the gravitational constant. It will make little difference what probable value is taken for $(D\text{-}\delta)$. Assuming $(D\text{-}\delta)$ be equal to 0.1 and substituting,

$$F = \frac{4}{3} \times 3 \times (4 \times 10^{-4})^3 \cdot 10^{-1} \times 10^3$$
$$= 2 \cdot 5 \times 10^{-8} \text{ dynes.}$$

It follows then, that the gravitational component is negligible and that the force, 10^{-5} dynes, the "adhesive force," of this single pseudopod was at least of this order of magnitude. Or assuming the area of the pseudopod on the capillary to be 1 sq. μ, the force per sq.mm. would be $(10^{-5} \times 10^6)$ or 10 dynes. This value is so small that the probability of its representing tensile strength rather than adhesive force is slight. The tensile strength of frog muscle, for example, is 6×10^4 dynes per sq.mm. (3).

From the foregoing it may be readily understood why leucocytes stick to a capillary wall with such apparent ease although a seemingly overwhelming force introduced by the blood stream is present. If

this phenomenon be repeatedly observed, it may be accepted that the adhesive force, of the order of magnitude of 10^{-5} dynes, is exerted by one pseudopod. A leucocyte touches the capillary wall with many more pseudopods. And since the force of the stream at the walls of the capillary is less than at the centre, a sufficient resistance may easily be offered by more than one pseudopod to produce leucocyte arrest and preparation for emigration.

SUMMARY AND CONCLUSIONS.

Observations on the behavior of a frog leucocyte *in vivo* have led to the conclusion that an adhesive force of at least 10^{-5} dynes may be offered by one pseudopod. This adhesive force, of one pseudopod, is of the order of magnitude sufficient to cause leucocyte arrest previous to emigration through the capillary wall.

I desire to express my thanks to Professor H. Freundlich for his kindness in reading the manuscript.

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THE STRUCTURE OF B. ANTHRACIS AND REVERSAL OF THE GRAM REACTION.*

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PLATES 34 TO 38.

(Received for publication, May 31, 1927.)

If a small amount of aqueous solution of acriviolet, gentian violet, or acriflavine be added to a thick aqueous suspension of a young culture of *B. anthracis* it will be found that after a longer or shorter interval of time a large proportion, if not all, of the organisms are changed by the exposure from sharply Gram-positive to sharply Gram-negative. The speed with which this change takes place varies with the strain of *B. anthracis* examined. In one strain studied, Gram reversal began in a few minutes and was complete within 2 hours. In other strains 19 hours were required to produce the change and even at the end of this period a few Gram-positive individuals were sometimes still found in the smears. The Gram-negative forms of *Bacillus anthracis* produced by exposure to these dyes are notably smaller in calibre than the Gram-positive forms, the difference in diameter as measured by means of a filar micrometer being in the neighborhood of 40 per cent.

That such changes could be produced in this organism by exposure to aniline dyes was first observed about a year ago. The present communication is concerned with studies made, since that time, of the reversal of Gram reaction, and of the change in calibre, which the dyes caused; and with attempts to interpret the significance of the two phenomena.

* Preliminary report of this work appeared in *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 737.

The Gram Stain.

In our early observations as to the effect of aniline dyes on *B. anthracis* the smears were stained by Paltauf's modification of the Gram method.[1] The findings thus obtained were also confirmed by the study of smears stained by the other well known modifications: Claudius',[2] Burke's,[3] Atkin's,[4] and Kopeloff's.[5] After the examination of about 2000 smears, each specimen being stained and examined by two observers, the definite conclusion was reached that Burke's modification was superior to all others. It was therefore, from that time on, exclusively used. The opinion as to its superiority was amply confirmed by the subsequent studies which involved the examination of nearly 12,000 smears. Whenever "Gram stain" is mentioned in this report it is the Burke modification (unless otherwise specified) to which reference is made. With the Burke technic the results are constant and absolutely clear-cut. The colors in the final smears contrast sharply (a bluish black with a pink) and one is never left in doubt as to whether an individual organism is to be called Gram-negative or Gram-positive.

When stained by the Burke technic young cultures of *B. anthracis* are definitely and constantly Gram-positive. An occasional Gram-negative individual may be seen, but these are *only* occasional and negative forms are usually entirely absent. The Gram-negative organisms, like *B. coli*, which are commonly used for controls in experimental work, are equally constant and sharp in their reaction to the Burke stain. If therefore the technic as described by Burke be carefully followed and the precautions observed which it is well known must be borne in mind in using the Gram method, the Burke modification is a method of exactness and from its results trustworthy conclusions can be drawn.

[1] Sharnosky, J., *Proc. New York Path. Soc.*, 1909, lx, N. S., 5.
[2] Claudius, M., *Ann. Inst. Pasteur*, 1897, xi, 332.
[3] Burke, V., *J. Bact.*, 1922, vii, 159.
[4] Atkin, K. N., *J. Bact.*, 1920, iii, 321.
[5] Kopeloff, N., *Lactobacillus acidophilus*, Baltimore, 1926, 204.

Exposure of Organisms to Aniline Dyes.

The technic of the experiments in which reversal of the Gram reaction of *B. anthracis* was produced was the following.

A heavy suspension of 4 hour culture of the organism was made in distilled water. ½ cc. of this suspension was placed in each of two tubes. To one of these tubes an oeseful of 1 per cent aqueous acriviolet was added. Smears were made at the beginning of the experiment and stained by Burke's method. These always showed the organism to be 100 per cent positive except that an occasional stray Gram-negative individual might be found. The tubes were put away at room temperature and smears from each tube examined at various intervals. It was found that Gram-negative forms began very soon to appear in the tube to which acriviolet had been added. In the case of the more rapidly changing forms, like No. 10,[6] reversal of Gram reaction began almost at once and was complete in 2 hours or less (Fig. 1). In the more slowly changing forms like N.Y.B. of H.,[7] 5 per cent of Gram-negative individuals appeared in 2½ hours.[8] At the end of 19 hours smears from the acriviolet tube showed that the organism had changed from a predominantly Gram-positive to a predominantly Gram-negative one. In other experiments reversal proceeded until about 75 per cent of the organisms had become Gram-negative and further change occurred very slowly if at all.

The first observations as to the effect of aniline dyes on *B. anthracis* were made with a strain furnished by the New York Board of Health. In order to determine that the reversal phenomenon was not peculiar to this particular strain of *B. anthracis* the experiment was repeated

[6] The numbers refer to the American Type Culture Collection.

[7] The strain referred to as "N. Y. B. of H." was isolated at the New York Board of Health Laboratories from a shaving brush in January, 1923.

[8] The roughness of a method in which percentages of Gram-positive and Gram-negative forms in a smear are estimated without actual counts is of course perfectly understood. Small variations are, under such conditions, of no significance whatever and no attention was paid to them. But the difference between a smear containing 99 per cent positive forms and one containing only 30 per cent positive forms is of course obvious at a glance and it was only differences of this order which were taken into account. In all cases the smears were stained by two observers and each smear recorded by both; the figures of the two records agreed pretty closely. It was found that the changes produced by the dyes were the same even when varying amounts were added and exactness of dilution was not necessary; "1 oeseful of 1 per cent dye" was therefore a sufficiently accurate measure. The oese used measured 2.33 mm. internal diameter.

with Strains 8, 9, 10, 240, 241, and 242 of the American Type Culture Collection. The authenticity of all of these strains was established by animal inoculation. It was found that the reversal phenomenon was produced in each instance by the exposure of the organism to aniline dyes so that the conclusion seemed warranted that the reaction was characteristic for the genus.

Similar experiments were then undertaken with other members of the spore-bearing, aerobic group. The first group studied was *B. subtilis* of which six strains were examined: one strain, isolated from hay, in our own laboratory and Nos. 102, 465, 466, 243, and 3 A. T. C. These experiments with *Bacillus subtilis* showed that a reversal of the Gram reaction similar to that of *B. anthracis* results from exposure to aniline dyes; but the phenomenon was less striking, since in some of the strains a moderate degree of reversal also occurred in the control tubes.

The definite findings in the case of *B. anthracis* and the suggestive findings in the case of *B. subtilis* suggested that the phenomenon might be common to all sporogenous aerobic[9] bacteria and a study was therefore made of this whole group, the strains being obtained from the American Type Culture Collection. The following organisms were examined.

B. mesentericus	No. 76
" *megatherium*	" 72
" "	" 71
" *mycoides*	" 270
" " *roseus*	" 80
" *adhærens*	" 271
" *niger*	
" *agri*	
" *laterosporus*	
" *simplex*	
" *globigii*	
" *albolactus*	
" *aterrimus*	
" *panis*	
" *prausnitzii*	
" *ruminatus*	
" *flavus*	" 58

[9] Nothing can at present be said about the behavior of anaerobes.

With a number of these organisms, reversal of Gram positivity was produced just as in the case of *B. anthracis*. This was true for example of *B. megatherium* and *B. mycoides*. But in the case of the majority of strains in this group either the results were inconstant or reversal occurred in the control tubes, so that no statement can be made as to the existence of any general relation between reversal phenomenon and the presence of spores in bacteria.

The investigation was then extended by making a study of non-spore-bearing Gram-positive organisms. Experiments were done with the following 50 strains.

	No.		No.
Sarcina lutea	272 A.T.C.	*Actinomyces maduræ*	552
" *rosea*	188	" *bovis*	549
" *aurantiaca*	146	" *gypsoides*	550
" *flava*	147	*Staphylococcus epidermidis*	155
Micrococcus auranticus	387	" *albus*	251
" *luteus*	379	" *citreus*	395
" *freudenreichii*	407	" *aureus*	477
" *flavus*	140	" *candicans*	154
" *conglomeratus*	401	" *tetragenus*	159
" *flavoroseus*	397	*Bacillus xerosis*	373
" *cereus*	394	" *diphtheriæ*	475
" *varians*	399	" *hoffmanii*	371
" *ureæ*	408	*Staphylococcus pyogenes*	160
Rhodococcus ruber	534	*Streptococcus lactis*	558
" *rhodochrous*	184	*Lactobacillus casei*	334
" *roseus*	185	*Staphylococcus* (No. 33)	Torrey
" *cinnabareus*	514	" (Richards)	"
Actinomyces asteroides	322	" (No. 77)	"
" *hominis*	551	" (isolated by Churchman)	

B. diphtheriæ	N. Y. B. of H.
" "	" " " " "
" "	" " " " "
B. hoffmanii	" " " " "
Pneumococcus Type 1	" " " " "
" " 2	" " " " "
" " 3	" " " " "
" " 1	Rockefeller Institute
" " 2	" "
" " 3	" "
B. tuberculosis bovine	N. Y. B. of H.
" " "	" " " " "

This study of the behavior toward aniline dyes of non-spore-bearing Gram-positive bacteria showed, that in striking contrast to the results produced in the case of *B. anthracis* and other members of the spore-bearing group, the dyes mentioned were without any effect on the Gram positivity of the majority of the non-spore bearers. The experiments with certain of these organisms (about 15 strains) were, however, unsatisfactory and from them no conclusions could be drawn. Pneumococcus for example proved too fragile for this type of experiment and we were unable to get constant results whether we worked with blood agar cultures or with the peritoneal fluid from injected mice. Nor were we able to draw positive conclusions from the experiments with *Streptococcus lactis*, nor with *Actinomyces hominis* or *Actinomyces maduræ*. But for the majority of the organisms (about 35 strains) in this group the dye was absolutely without effect on the Gram reaction, even after many days exposure and even when the tube was heated in order to make the test more severe. The difference between the behavior to aniline dyes of *B. anthracis* and a number of the Gram-positive non-spore bearers was well shown by an experiment in which a suspension containing both *B. anthracis* and *M. freudenreichii* was used. Both of these organisms are definitely Gram-positive and the smears made at the beginning of the experiment showed nothing but Gram-positive forms. Smears made (at the end of 19 hours) from the tube to which acriviolet had been added showed that none of the individuals of *M. freudenreichii* had been changed but that about 95 per cent of the individuals of *B. anthracis* had become Gram-negative. The smears from the control tube at this time showed all the organisms of both strains to be still Gram-positive (see Fig. 2).

It seems clear from the experiments just described, in which the bacterial field was pretty completely covered so far as the Gram-positive aerobic forms are concerned, that two types of organisms may be distinguished, one of which exhibits reversibility of Gram reaction when exposed to acriviolet and the other of which remains Gram-positive in spite of long exposure to the dye. The organisms which exhibit the reversal phenomenon in clear-cut fashion belong in the spore-bearing group; the organisms which resist this effect of the

dye belong in the non-spore-bearing group.[10] This fact is sufficiently striking and may be of significance. There are, however, numerous exceptions in both groups, so that one cannot definitely relate the reversal phenomenon to the presence or absence of sporogenic material.

Other Dyes.

It has been said that the reversal phenomenon is brought about by exposure to acriviolet and this dye did appear to be more effective than any other in producing the result. But exposure to other dyes, notably acriflavine, gentian violet (both Grübler, and Coleman and Bell), and ethyl violet also resulted in a reversal of Gram reaction and a diminution of calibre.

Other Substances than Dyes.

Since the dyes are known to have a bacteriostatic effect upon certain of the organisms which exhibit reversal phenomenon experiments were done with other substances, not dyes, also capable of producing bacteriostasis. In these experiments both *Bacillus anthracis* and *Staphylococcus aureus* were exposed to the action of alcohol, formalin, and Zenker's fluid. These substances produced no effect on the Gram reaction of either organism.

Relation of Reversibility to Acidity.

The dyes used, though basic or neutral in name, have a pH between 3 and 4[11] and this fact raises the question also suggested by the work of Deussen[12] (who noted the effect of acids on the Gram reaction—but not on the size—of certain Gram-positive organisms), whether the phenomenon here reported might not be due to the slight acidity

[10] Deussen (Deussen, E., *Z. Hyg. u. Infectionskrankh.*, 1918, lxxxv, 235) noted a similar difference in the effect of certain substances on the Gram reaction of different Gram-positive organisms.

[11] The potentiometer readings are as follows:

1 per cent aqueous gentian violet..... pH 3.5
" " " " acriflavine..... pH 3.5

[12] Deussen, E., *Z. Hyg. u. Infectionskrankh.*, 1918, lxxxv, 235. This author regarded the phenomenon as a purely chemical one and thought the effect of acids to be in direct proportion to their degree of dissociation.

(it is very slight) of the bacterial suspension resulting from the addition of the small amount of 1 per cent acriviolet or gentian violet used.[13] Since the result produced by these dyes was not produced by acid fuchsin (pH 2.55) it seemed unlikely that acidity of dye was the whole explanation of the phenomenon. That the addition of strong acids, at least, did not produce reversal of Gram reaction nor diminution of calibre was easily shown by the following experiment.

Three tubes each containing ½ cc. bacterial suspension (*B. anthracis* N. Y. B. of H.) were prepared. To the first was added 1 oeseful of 1 per cent acriviolet, to the second 1 oeseful of 10 per cent HCl, to the third 1 oeseful of concentrated HCl. Complete reversal took place in the acriviolet tube within 19 hours. In the acid tubes no change was observed at the end of 19 hours, and only slight change at the end of 48 hours.

A second experiment to test this point was done by adding to a series of tubes containing the bacterial suspensions 1 oeseful of water which had been brought to pH 1.2, 1.8, 2.4, 3, 3.8, and 7. In none of these tubes did reversal occur, although complete reversal took place in the control tube containing dye. This experiment is open to the objection that the acid used was not buffered and therefore its action may not have been continuous. In the dyes, on the other hand, acid may be present in just the right proportion and it may be so buffered as to act continuously. Efforts to test this point out by buffering with KCl and with KH phthalate, the acid added to the bacterial suspensions were defeated because it was found that the buffers themselves led to some reversal. The point must, therefore, be left for the present in abeyance.

Modifying Factors.

The question of the relation of the reversal phenomenon to the age of the culture was investigated. This was done by making bacterial suspensions of agar growths, at intervals of 1 hour, from 1 hour up to 7. Though the results of these experiments were not absolutely constant, there is reason for believing that very young cultures are more resistant to the reversing effect of acriviolet than older cultures.

The question of the nature of fluid in which the suspension is made and its relation to the reversal phenomenon was studied by making

[13] The pH of the distilled water used in this laboratory is 6.0. Potentiometer readings of ½ cc. of this water containing 1 oese 1 per cent acriviolet showed a pH of 5.3+.

suspensions in distilled water, in tap water, and in physiological saline, and adding dye to these tubes. Reversal proceeded more rapidly in the water tubes than in saline.

Reduction in Calibre of Bacteria by Exposure to Dyes.

Reference thus far has been made only to the change in Gram reaction produced by exposure to acriviolet. But another equally striking, if not more striking, change has also been observed; for it is noted in the smears of *B. anthracis* which have been exposed to acriviolet that the Gram-negative forms are much smaller in transverse diameter than the Gram-positive forms. This difference in size is so striking, the organisms stained with safranine being quite slender and those stained with methyl violet quite stout, that one might easily conclude that the smears contained a mixture of two different organisms, one a robust Gram-positive and the other a slender Gram-negative (see Fig. 1, *B*). In smears made when the change is about half complete these pink and bluish black segments may alternate in the same chain giving a most striking appearance (see Fig. 1, *B*, and Fig. 5, *A–D*). It will be noted that when spores are present they are entirely contained within the Gram-negative forms. This is apparent in many of the illustrations; but particular attention is called to the organisms marked *a* Fig. 1, *B*, and *a* Fig. 3, *A*.

Large numbers of measurements of the Gram-positive and Gram-negative forms were made, a Zeiss filar micrometer being used for the purpose. These measurements showed the Gram-positive forms to be practically always larger in transverse diameter than the Gram-negative forms. The difference in size is of course not absolutely constant in degree, varying from .123μ to .962μ; but in only one instance has a Gram-negative segment of a bacterial chain been seen which was larger than the adjacent Gram-positive segment, and in only the most occasional instances were they of the same calibre. Comparative measurements of 25 Gram-positive and 25 Gram-negative adjacent segments from a smear of *B. anthracis* No. 10 stained by Burke showed: average diameter of the Gram-positive forms 1.2236μ, of the Gram-negative forms, .7091μ, a difference of .5145μ or 42+ per cent.

In the complete study 292 organisms were measured. The average diameter of the Gram-positive forms was 1.112μ; of the Gram-negative forms .686μ. This gave an average difference of .426μ or 38.3 per cent.

When measurements were made of specimens which had been stained by the

Paltauf modification, the same general facts came to light although the difference between the Gram-negative and Gram-positive forms was not quite so marked as in specimens stained by Burke's method (see Fig. 3, *A* and *B*, and Fig. 5, *D*). In this set of observations, measurements were made of 50 organisms. The average transverse diameter of the Gram-negative forms was .311432μ smaller than that of the Gram-positive forms, a reduction of 29.74 per cent in diameter.

Exposure to acriviolet therefore not only causes *Bacillus anthracis* to change its Gram reaction from sharply Gram-positive to sharply Gram-negative; it also leads to a reduction, by as much as 40 per cent or even more, in its transverse diameter. Whether a similar change in longitudinal measurement also occurs it is impossible to say with certainty since the variation in length of the individuals in a given smear of *B. anthracis* is usually so great as to make comparative measurements impossible. But Gram-positive material may be shown to exist between the Gram-negative segments of a chain of *B. anthracis* and when this is removed from the ends of the organisms by excessive decolorization of lightly stained specimens diminution in length must occur (see Fig. 4, *A*, *a*, *b*, and *c*, and *C*, *a* and *b*).

Significance of Reversal.

What is the significance of the change in Gram reaction and the diminution in size produced by the exposure of *B. anthracis* to acriviolet? It is clearly not a specific reaction since it is produced also by substances other than dyes and indeed occurs in a few individuals even in the control tubes. Since acriviolet is known to have a marked bacteriostatic effect on *B. anthracis*, the reversal produced by this dye might be thought to be purely a death phenomenon. This, however, seems unlikely to be the case since other organisms (like *M. freudenreichii*) which are killed by acriviolet do not exhibit reversal on exposure to this dye, and since—if the vegetative forms of *B. anthracis* be killed in other ways (as by prolonged boiling or exposure to bichloride of mercury)—reversal is not produced. Since a certain amount of reversal usually occurs sooner or later in the control tubes to which no dye has been added, it is not unlikely that the dye simply promotes or hastens a change in the bacteria, caused by degeneration or lysis (dependent perhaps on change of reaction), which would occur without any dye. Bacteria are no longer thought of as entities

with permanent characteristics, but biological units which change constantly. It is now well known that in their cycle of growth they both lose and acquire characteristics; the Gram reaction is one of the characteristics which may change during growth or be changed by environment. No one has ever demonstrated an anatomical basis which might explain this change of Gram reaction; in the case of *B. anthracis* the finer structure of the organism as revealed by these studies appears to offer such an anatomical basis.

But what is the cause of the change in size? Three explanations suggest themselves. It might be thought that the change in size was apparent rather than real and that it resulted from the heaping up of methyl violet on the surface of the Gram-positive segments, a heaping up which did not occur in the Gram-negative segments whose diameter appeared therefore to be less. It is possible of course that the apparent size of the stained bacteria is greater than the actual size of the living organisms but I am inclined to think that the difference in size produced by staining is not great. In any case the suggestion that the observed difference in size between Gram-negative and Gram-positive segments is not real but entirely dependent on accumulations of stain in some segments which do not occur in others is excluded by the fact that the difference in size is to be observed (under the limitation noted below) also in hanging drop specimens—which have not been stained at all—as well as in specimens stained by safranine alone, by fuchsin alone, by Burke's methyl violet alone, and by saturated alcoholic gentian violet alone (Fig. 3, *C* and *D*).

The second explanation which suggests itself is that the difference in size between adjacent Gram-positive and Gram-negative segments is due to shrinkage. It is conceivable that when dye is added to an aqueous suspension of *B. anthracis* osmotic forces, acting through the bacterial membrane, extract material (possibly water) from certain segments, whose size is in this way diminished. Since the weighing experiments were done with desiccated specimens, dehydration could not be the cause of the loss of weight in the Gram-negative segments.

If dehydration be brought about by exposing the bacteria to 95 per cent, followed by absolute alcohol no such picture is obtained as follows exposure to the

dyes. A slight diminution in diameter does occur, as the following measurements show, but the Gram reaction is not reversed.

Diameter control organisms (15 measured)	1.06 μ
" after 48 hours in 95 per cent alcohol	1.00 μ
" " 24 " additional absolute alcohol	.85 μ
Total loss of diameter	.21 μ
	20 per cent

If a bacterial suspension be evaporated to dryness and complete desiccation thus produced smears for examination of the stony material which results can only be obtained after grinding it in a mortar. This vigorous treatment destroys bacterial morphology and no satisfactory observations can be made on the debris.

A third explanation of the phenomenon is that the difference in size observed in the hanging drop as well as in stained specimens is a real difference and that it is to be explained by the fact that *B. anthracis* is composed of two parts, an outer layer which is Gram-positive, and an inner core which is Gram-negative. According to this explanation, when the proper dye is added to a suspension of *B. anthracis* the outer Gram-positive coat is destroyed and only the inner Gram-negative core remains. Ectoplasm and endoplasm might be the most correct terms to apply to these portions of the bacterial structure if they had not already been used by Zettnow[14] in a somewhat different sense. Cortex and medulla are convenient descriptive terms although usually applied to an organ rather than an organism. Adopting these terms tentatively we may say—if the suggested hypothesis as to difference in size is correct—that the Gram-positive cortex of *B. anthracis* is rather easily destroyed by lysis and by degeneration; that it undoubtedly often disappears during bacterial growth (which accounts for the occasional presence of Gram-negative forms of *B. anthracis* in smears from cultures and their frequency in old cultures); that the cortex is susceptible to the action of distilled water, so that it will disappear in this medium after prolonged exposure; that it is notably sensitive to the action of certain dyes; and that when the cortex is thus destroyed the slender Gram-negative medulla comes into view.

[14] Zettnow, *Z. Hyg. u. Infectionskrankh.*, 1899, xxx, 1; 1918, lxxxv, 17.

A good deal of evidence has been gathered for the correctness of this hypothesis, but it cannot perhaps be said that the case is absolutely established. Final proof would be afforded if cross-sections of bacteria could be obtained and if these, when stained by Burke's method, exhibited a Gram-negative core and a Gram-positive periphery. This proof, though we have endeavored to obtain it, cannot now be presented at least in convincing form. Numerous efforts have been made by varying technic to get satisfactory bacterial sections but, largely because of technical difficulties, without success. In the preparations examined a few objects were seen which strongly suggested cross-sections of bacteria, pink in the centre and blue at the periphery. But in examining these preparations, with the high-power and strong illumination required, color distortions occur—particularly in looking at minute objects—and these may easily lead one astray. Further attempts of this kind are now in course.

Perhaps as strong evidence as could be desired for the hypothesis that *B. anthracis* consists of a Gram-positive cortex and a Gram-negative medulla (short of that which bacterial sections would furnish), is provided by the picture obtained when smears from a young culture of this organism are stained by a modified Burke technic in which duration of exposure to dye and mordant is greatly shortened, and duration of exposure to decolorizer greatly increased. In specimens of this kind one finds individual bacteria in all stages of decolorization. A few of them may have retained their Gram positivity. But many are entirely Gram-negative, while still others show the remains of Gram-positive material clinging to the surface in the form of granules or lumps or plaques among which the pink Gram-negative medulla can be seen shining through. The appearance is exactly that of a banana, the skin of which had been in places removed, partially exposing the fruit within (see Fig. 4, *A*). Such a picture fits in well with the hypothesis under discussion.* Whether in specimens which have thus been partially decolorized bacterial substance is actually dissolved away from the surface of the organism by the

* A. T. Henrici (*J. Med. Research*, 1914, xxx, 409) has described similar partial decolorizations of yeast cells but appears not to have drawn any conclusions as to the significance of these observations beyond their bearing on the Gram reaction.

decolorizer, or whether merely the mordanted methyl violet is dissolved away leaving the now unstained and invisible bacterial surface material behind, has not yet been definitely proven. The former explanation seems perhaps more likely to be the correct one. If it is, then specimens which have been partially decolorized ought—if restained by the ordinary Burke technic—to look just as they did before the restaining, since the surface material, capable of retaining the methyl violet, has been in large part removed by the partial decolorization. Such a result is illustrated in Fig. 4, *C*. The only difference between this picture, which represents a specimen which has been partially decolorized and then restained by Burke and the picture shown in Fig. 4, *A*,—which represents a specimen that has been partially decolorized but not restained—is that the Gram-negative material in Fig. 4, *C*, has a slight purplish tint. If such a result had been always obtained when this experiment was done the matter would be clear enough. But sometimes the partially decolorized organisms, when restained, retained the Gram stain throughout.

Two critical experiments could be done to determine whether the change of size, in the bacteria which have been exposed to dye, actually rested on the anatomical structure of *B. anthracis* as we have suggested. One ought to be able to show an actual loss of weight in the bacteria which have been subjected to the dye, as compared with the controls; the demonstration of such a loss of weight, provided the specimens be desiccated before weighing, would prove that apparent diminution in size was real and would eliminate shrinkage as an explanation of the phenomena. One ought also to be able to demonstrate the presence of protein or hydrolytic products of protein in the acriviolet to which the bacteria had been exposed. These two tests were made and since both gave definite results agreeing with the hypothesis they provided additional testimony that the explanation suggested was correct.

Weighing Experiments.

The weight of the desiccated centrifugate from a heavy aqueous suspension of *B. anthracis* was determined and compared with the weight of a similar centrifugate from a suspension to which gentian violet had been added and in which reversal of Gram and diminution

in calibre had been produced. Definite loss of weight was shown to have occurred in the gentian violet suspension. These experiments gave the following results.

	Control	*Gentian violet tube*
Weight of tube plus dried centrifugate...	11.350 gm.	11.8250 gm.
" " empty tube.....	11.324 "	11.8114 "
" " bacteria.....	.027 "	.0136 "
	.0136 "	
Loss of weight in gentian violet tube.....	.0134 "	= 49.6 per cent

If it be assumed that all the gentian violet dissolved in the tube went to the bottom with the bacteria during centrifugation the figures become (since the amount of gentian violet used weighed .001 gm.):

Weight of bacteria in control tube.....	.027 gm.
" " " " gentian violet tube.....	.0126 "
Loss of weight in gentian violet tube.....	.0144 " = 53.3 per cent

This experiment was repeated by identical technic except that on this occasion it was necessary to allow the tube, to which gentian violet had been added, to stand only 3 hours in order to produce a complete reversal of Gram reaction. The results of this experiment were as follows:

	Control	*Gentian violet tube*
Weight of tube plus dried centrifugate...	11.4535 gm.	11.0547 gm.
" " empty tube.....	11.3336 "	11.0274 "
" " bacteria.....	.1199 "	.0273 "
	.0273 "	
Loss of weight in gentian violet tube....	.0926 "	= 77.2 per cent

The experiment was again repeated in another way. Instead of centrifugating the specimens they were passed through previously weighed Berkefeld N 5 filters, which were then dried and weighed. The results were as follows:

	Control	*Gentian violet tube*
Weight of filter plus dried bacteria.....	64.1370 gm.	74.9680 gm.
" " " alone.....	61.5384 "	73.3028 "
" " bacteria.....	2.5986 "	1.6652 "
	1.6652 "	
Loss of weight in gentian violet suspension	.9334 "	= 35.9 + per cent

Protein Tests of the Filtrate.

If the suggestion is correct that the difference in diameter between the Gram-positive and Gram-negative forms of *B. anthracis*, is due to the solution of an outer Gram-positive coat, the presence of this protein—or protein-like—material ought to be demonstrable in the filtrate of a suspension of *B. anthracis* to which dye has been added. Such a demonstration was made. For the purpose the ninhydrin test was used. This test is not specific for proteins[15–17] so that it can only be said—not that the presence of protein was demonstrated—but that ninhydrin-positive bodies were present in the filtrates from suspensions of *B. anthracis* which had been exposed to dyes. These experiments were done as follows:

Heavy aqueous suspensions of *B. anthracis* were centrifugated, washed three times in saline, and filtered through unused Berkefeld filters into glassware which had been cleansed with aqua regia. Filtrates were tested by ninhydrin, biuret, and Heller tests. The control filtrates were negative. The filtrates from suspensions to which dye had been added gave a positive ninhydrin test, and, when concentrated by evaporation, a positive biuret.

It seems clear therefore that material, probably but not certainly protein in nature, is dissolved away by gentian violet, since its presence can be determined in the filtrate from suspensions of *B. anthracis* to which the dye has been added, the controls (made with saline in order to avoid bacteriolysis) being negative by the biuret and ninhydrin tests. Experiments are now under way to determine the nature of the material present in the gentian violet filtrate and to see whether it has any toxic, immunizing, or other important properties.

Many observers have noticed the occurrence, in cultures of *B. anthracis* (and indeed of other organisms), of bizarre forms. Some of these bear a remote similarity to those produced by exposure to dyes. But they have usually been dismissed as "involution forms" resulting from plasmolysis or plasmoptysis (*e.g.* Gotschlich),[18] or as

[15] Ruhemann, S., *J. Chem. Soc.*, 1910, xcvii, 2030; 1911, xcix, 798.

[16] Neuberg, C., *Biochem. Z.*, 1913, lvi, 500–506; 1914, lxvii, 56.

[17] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1916, xxv, 350.

[18] Gotschlich, E., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, i, 45.

"teratological forms,"[19] or as "degeneration forms." In his beautiful studies of the morphology of *B. anthracis* Preisz[20] pictures, without apparently paying much attention to them, bizarre forms of this kind. He worked with old cultures, the organisms were not stained by Gram, but vitally stained with fuchsin, and nothing is said of their Gram reaction. In Fig. 7, Table 3, of his 1909 publication stout and slender segments are represented. Nothing is said about them. The specimen was not stained by Gram. It was made from a 2 days old culture which had been exposed to a mixture of dog and guinea pig serum (5:1), and Preisz appears to have paid little attention to the question of size. He made no measurements. Preisz' chief interest lay in the capsule which he regarded as a degeneration product of the cell membrane. Zettnow,[14] who was interested chiefly in bacterial chromatin, worked solely with the Romanowski stain. He described the bacillus of anthrax as composed of two parts: an ectoplasm, which remains colorless by the Romanowski and all ordinary staining methods, and an entoplasm which stains blue by Romanowski, and which contains chromatin (stained red by Romanowski). "A large number of bacteria consist entirely of chromatin. Even in those which stain both blue and red, chromatin strongly predominates. In exceptional cases in very young cultures the plasma occurs in larger amounts than the chromatin but as growth proceeds the relation is reversed." The ectoplasm, compared to the entoplasm, is said to be relatively poor in water content and is more concentrated, as is clear from its greater resistance to stains, to plasmolysis, and to destructive influences. In contradistinction to *B. anthracis*, cocci and sarcinæ take only one color, the blue (entoplasm). Zettnow made no studies by the Gram method. He made no measurements. In our own work smears of organisms which had been exposed to acriviolet until partial reversal had occurred were stained by numerous polychrome methods, including Romanowski. No selective staining, such as that described by Zettnow, was observed. From all these data it seems clear that there is no relation between the picture obtained by Zettnow and that described in this communication. Zettnow's ectoplasm is said to remain colorless

[19] Maassen, A., *Arb. k. Gsndhtsamte*, 1904, xxi, 385.

[20] Preisz, H., *Centr. Bakt., 1. Abt., Orig.*, 1903–04, xxxv, 657; 1909, xlix, 341.

in all ordinary staining methods. Since both cortex and medulla stain readily both would appear to lie within the entoplasm.

Gram Stability and Gram Instability.

The fact that the material responsible for the Gram positivity of *B. anthracis* appears to be confined to the surface of this organism and that the centre is Gram-negative, made it seem likely that the Gram positivity of *B. anthracis* would be relatively unstable as compared with that, for example, of *M. freudenreichii*, an organism in which no distinction between Gram-positive surface and Gram-negative interior had been demonstrable. It had been found, indeed, by preliminary observations that,—although young cultures of *B. anthracis* were completely and definitely Gram-positive (when stained by the Burke method), provided the technic was accurately followed—an increasing proportion of individuals in a given specimen became Gram-negative if the time of exposure to dye and mordant was diminished and the time of exposure to decolorizer increased. A systematic study was therefore made to see what the effect on different Gram-positive organisms would be, of variations in time of exposure to stain, to mordant, and to decolorizer. It was found that for many organisms (*M. freudenreichii* may be cited as a typical example) variations in time of exposures made, within wide limits, practically no difference in the results, *M. freudenreichii* being always 99 per cent to 100 per cent positive. In the case of other Gram-positive organisms (*B. anthracis* for example), wide variations in the result could be obtained by varying the technic as regards time of exposure.[21]

The facts are well represented in Fig. 4, *B*, in which are illustrated the results of an experiment done with a suspension containing a mixture of *B. anthracis* (N. Y. B. of H.) and *M. freudenreichii*. The smear has been stained by a modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 10 minutes). *B. anthracis* has been largely decolorized: *M. freudenreichii* unaffected.

When investigated in two ways therefore—by the results of exposure to acriviolet (see Fig. 2) or by modifications of staining tech-

[21] Churchman, J. W., *Stain Technol.*, 1927, ii, 21.

nic in the direction of diminished time of exposure to dye and mordant and prolonged time of exposure to decolorizer (see Fig. 4, *B*)—two Gram-positive organisms are seen to behave quite differently, as regards their Gram reaction. Light is thus thrown on the mechanism of the Gram reaction and the fact emerges that this mechanism is not necessarily the same in the case of bacteria of different species even though their behavior to the standard technic is identical. This distinction between stable and unstable Gram-positives appears to be a useful one. It is suggested that in recording the Gram reaction of new species, some such set of comparative tests as here described be carried out so that the stability of the reaction be known.

Examination of Hanging Drop Specimens.

Attention has been called to the facts that the difference in size between Gram-positive and Gram-negative segments of *B. anthracis* is demonstrable in unstained hanging drop specimens, made from suspensions which have been exposed to acriviolet or gentian violet and in smears stained with a single dye like methyl violet. From these facts—represented in Fig. 3, *C* and *D*,—one can only conclude that the reduction in calibre is real and not the result of an artefact.

The experiment of May 25, from which Fig. 3, *D*, was made, may be cited. A suspension of a 3 hour culture of *B. anthracis* No. 10 was made and gentian violet added. At the end of 2 hours smears stained by Burke, showed that the organism had changed from 100 per cent Gram-positive to 50 per cent Gram-positive and beautiful instances of great variation in size between adjacent bacterial segments were numerous. A hanging drop specimen was made. The organisms had picked up enough of the gentian violet to which they had been exposed to be clearly visible and the marked differences in size represented in the figure were readily made out. It was noticeable that the stout segments were always more deeply stained than the slender.

Observations of this sort left no doubt as to the reality of the difference in size between adjacent bacterial segments. But throughout the experiments with hanging drop specimens I was struck by a curious fact: If specimens of *B. anthracis* which had been

treated with acriviolet were stained by Burke or by Paltauf and the stout and slender forms measured, a difference of diameter—often as high as 60 per cent—was readily made out. If, however, specimens from the same tube were stained with a single dye or examined in the hanging drop this difference in size was sometimes by no means so readily observed. This observation led us at first to the conclusion that the difference in diameter which we had seen in the smears stained by Burke was apparent and not real. The demonstration, however, of an actual loss of weight, of a clear difference of size in many of the hanging drop specimens, of the presence of protein (or related substance) in the filtrate from bacterial suspensions containing acriviolet, and of the presence of cortex and medulla, by methods of partial decolorization, all pointed to a real and not an apparent loss of calibre.

The fact that the difference in size between Gram-positive and Gram-negative segments is not as a rule so obvious, in the fresh specimen and in those stained with a single stain, as it is in the specimen stained by Burke, is to be explained partly by the fact that the Burke stain gives a contrast of color which at once attracts the eye and at the same time emphasizes the difference in size. This contrast is absent in the single stains and change in size unless very marked is easily overlooked even when it is present. Another reason for the apparent discrepancy is that the Gram-negative segments—that is to say, the smaller segments—often stain (when only single stains are used) very lightly, in some cases not at all; and these ghosts easily escape detection. In the fresh specimen the slender segments are translucent, often nearly invisible, and it is probable that many of them are overlooked. It may also be the case that the Burke stain somewhat exaggerates the degree of the difference in size, which is none the less real.

It results from all this that one must have for examination in the fresh or with single stains, specimens in which the difference in size is quite marked indeed. In specimens stained by Burke on the other hand the difference in size is very striking even when it is not great in degree.

SUMMARY.

1. The addition of small amounts of aqueous gentian violet, acriflavine, or acriviolet to suspensions of young cultures of *B. anthracis* reverses their Gram reaction, and diminishes their diameter about 40 per cent.

2. The time required for these changes varies with the strain of *B. anthracis* examined.

3. These changes are accompanied by a loss of weight.

4. Ninhydrin-positive substances are demonstrable in the filtrate from suspensions of *B. anthracis* to which dyes have been added.

5. Similar changes are produced by these dyes in many, but not in all, of the sporogenic aerobes.

6. Non-spore bearers are for the most part unaffected in these ways by the dyes, although to this statement there are a number of exceptions.

7. The change in size produced by the dyes is demonstrable in hanging drop specimens as well as in stained smears, but not with equal constancy.

8. Partial decolorizations of *B. anthracis* are described, which are produced by modifications of the Burke technic in which time of exposure to dye is shortened and time of exposure to decolorizer lengthened.

9. The explanation for these phenomena which accords with all the known facts is that they depend on the existence in *B. anthracis* of a Gram-positive cortex and a Gram-negative medulla. Positive proof of the correctness of this explanation must await the evidence furnished by cross-sections of bacteria.

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Camera lucida drawing. Magnification × 4200. This plate shows two successive stages in the change produced in the Gram reaction and in the calibre of *B. anthracis* (A. T. C. No. 10) by exposure to acriviolet. Smears stained by Burke's method.

A, smear made for control at the beginning of the experiment. The organism is completely Gram-positive.

B, smear made from a specimen which has been exposed to acriviolet for 45 minutes. Partial reversal of the Gram reaction has occurred, about 25 per cent

of the individuals being Gram-positive and 75 per cent Gram-negative. Notice the marked difference in calibre between the Gram-negative forms and the Gram-positive, and the fact that the spore is entirely contained in the Gram-negative portion of the organism (*a*). One gets the impression of a smear made from a mixture of two different organisms.

C, smear made from a specimen which has been exposed to acriviolet for 2 hours, showing complete reversal of Gram reaction. Practically every individual is now Gram-negative.

PLATE 35.

FIG. 2. Camera lucida drawing of a mixture of *B. anthracis* (N. Y. B. of H.) and *M. freudenreichii* (A. T. C.). Magnification × 4200. Stained by Burke's method.

A, smear from the control tube. Both organisms are sharply and completely Gram-positive. One stray Gram-negative individual of *B. anthracis* is seen.

B, smear from specimen which has been exposed to acriviolet. The Gram reaction of *B. anthracis* has been in large part reversed, almost all of the individual organisms being now Gram-negative. All the individual organisms of *M. freudenreichii* are, on the other hand, as strongly Gram-positive as at the start of the experiment. The difference in calibre between Gram-positive and Gram-negative forms is very evident.

PLATE 36.

FIG. 3. Camera lucida drawings. Magnification × 4200.

This plate represents the slight variations in difference of size, between Gram-positive and Gram-negative forms, produced by varying the conditions of the experiment.

A, smear made from a specimen of *B. anthracis* (N. Y. B. of H.) which has been exposed to acriviolet until partial reversal of Gram reaction has occurred, and *stained by Paltauf's modification*. The difference in calibre, between Gram-negative and Gram-positive forms, though definite, is less marked than in *B*. Notice (*a*) the spores within the medulla.

B, smear made from a specimen of *B. anthracis* (A. T. C. No. 10) which has been exposed to acriviolet and *stained by Burke's modification*. Compare the difference in calibre with that shown in *A*.

C, smear made from a specimen of *B. anthracis* (A. T. C. No. 10) which has been exposed to acriviolet and *stained only with Burke's methyl violet*. The presence of stout and slender forms is evident. The picture obtained in the specimens stained by the Burke technic cannot therefore be an artefact produced by that technic.

D, drawing of individual organisms seen in a hanging drop specimen made from a suspension of *B. anthracis* (A. T. C. No. 10) which has been exposed to gentian violet. The living organisms have taken up enough of the stain to make them clearly visible and the difference in calibre between Gram-positive segments

(which take the stain fairly well) and the Gram-negative segments (which remain pale) is perfectly clear. The difference in size caused by exposure to dyes is therefore real and not an artefact produced by the Burke technic.

PLATE 37.

FIG. 4. Camera lucida drawings. Magnification × 4200.

A, partial decolorization of *B. anthracis* (A. T. C. No. 10) by modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 3 minutes).

The Gram-positive material has been partially removed from the surface, persisting only as plaques or lumps or stippling through which the pink Gram-negative rod in the interior of the organism can be seen. Notice (*a*, *b*, *c*) the caps of Gram-positive material at the ends of the bacterial segments.

B, smear of a mixture of *B. anthracis* (A. T. C. No. 10) and *M. freudenreichii* (A. T. C.) which has been stained by modified Burke technic (stain 5 seconds, iodine 5 seconds, decolorizer 10 minutes).

Almost every individual organism of *B. anthracis* has been completely decolorized; but *M. freudenreichii* has remained completely Gram-positive.

C, smear of *B. anthracis* (A. T. C. No. 10) stained by the same modification of Burke's technic as that used in the experiment represented in *A*, and then restained by the ordinary Burke technic. The portions of the organisms which were decolorized by the first process now fail to take the stain in the second and appear as in *A*, except that the pink color has become purplish pink. Notice (*a*, *b*) terminal caps of Gram-positive material.

PLATE 38.

FIG. 5. Photographs of single chains from specimens of *B. anthracis*, which have been exposed to acriviolet, to show the difference in calibre between Gram-negative and Gram-positive segments as seen at various magnifications. On account of the difference in calibre accurate focussing at the higher magnifications is impossible.

A, magnification × 1400. Stained by Burke's modification. Alternating Gram-positive and Gram-negative segments. *B. anthracis* (A. T. C. No. 10).

B, magnification × 1800. A Gram-negative segment with a Gram-positive segment at either end is plainly seen. *B. anthracis* (A. T. C. No. 10) stained by Burke's modification.

C, magnification × 2500. The same chain represented in *B*.

D, magnification × 3200. *B. anthracis* (N. Y. B. of H.) stained by Paltauf's modification. The difference in calibre between Gram-positive and Gram-negative segments, though clear, is less marked than in *A*, *B*, and *C*.

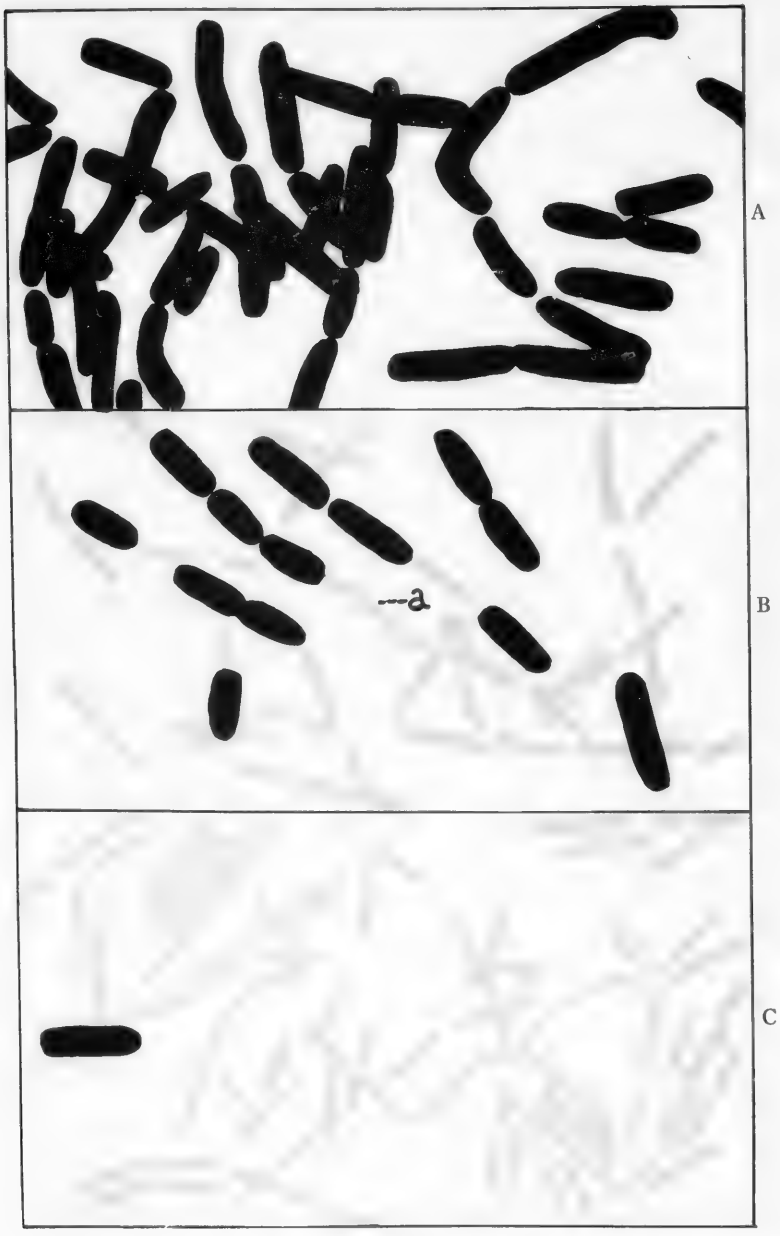

FIG. 1.

(Churchman: *B. anthracis* and Gram reaction.)

FIG. 2.

(Churchman: *B. anthracis* and Gram reaction.)

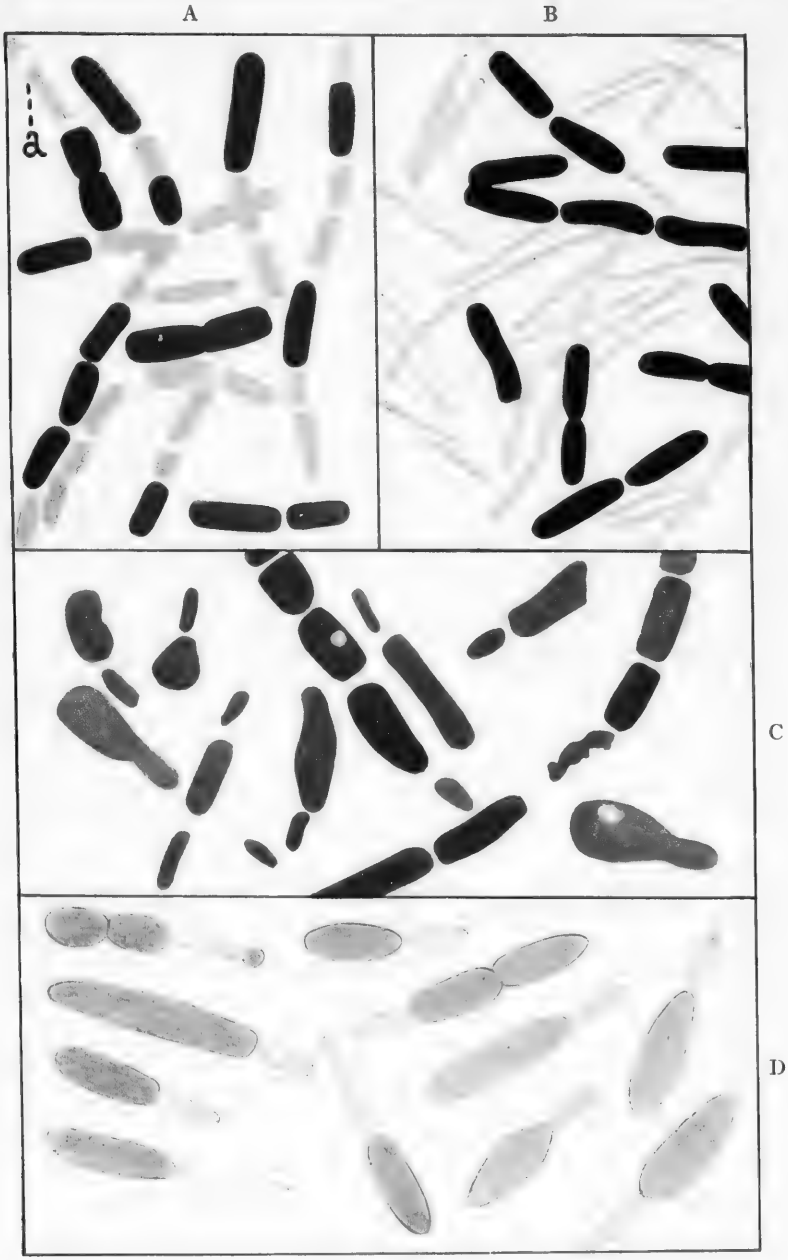

FIG. 3.

(Churchman: *B. anthracis* and Gram reaction.)

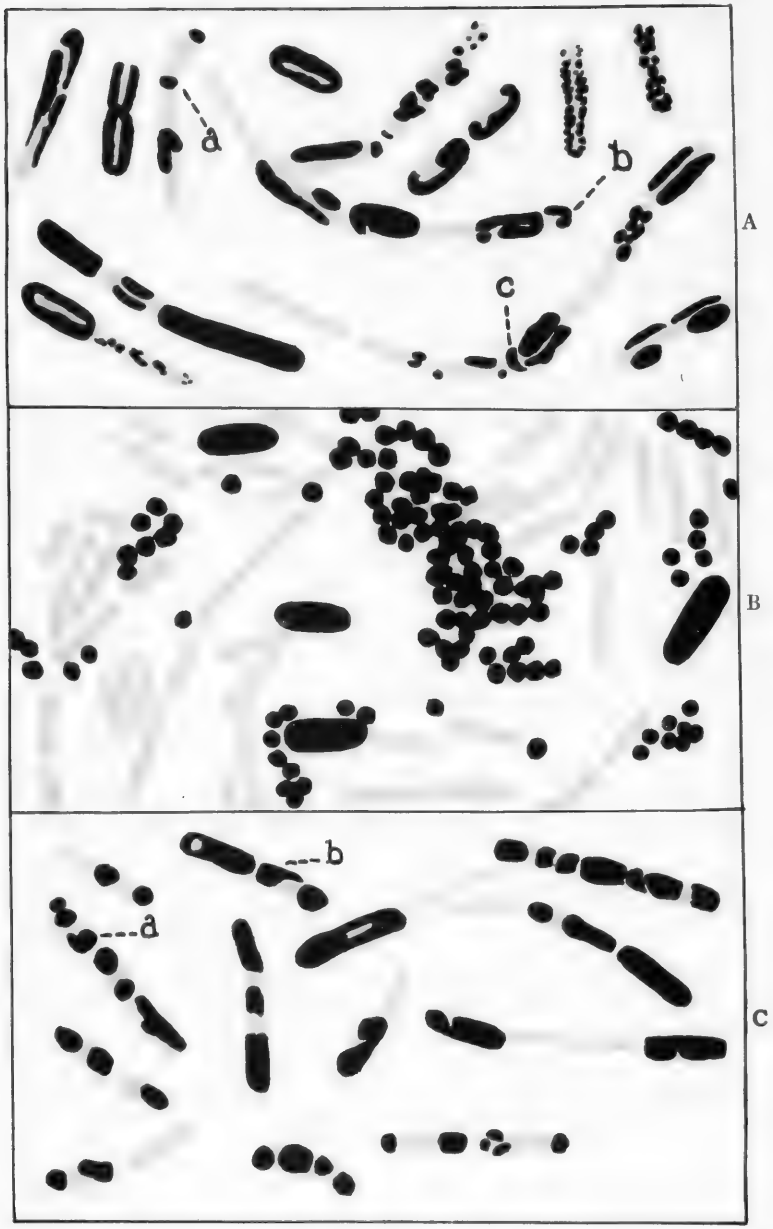

FIG. 4.

(Churchman: *B. anthracis* and Gram reaction.)

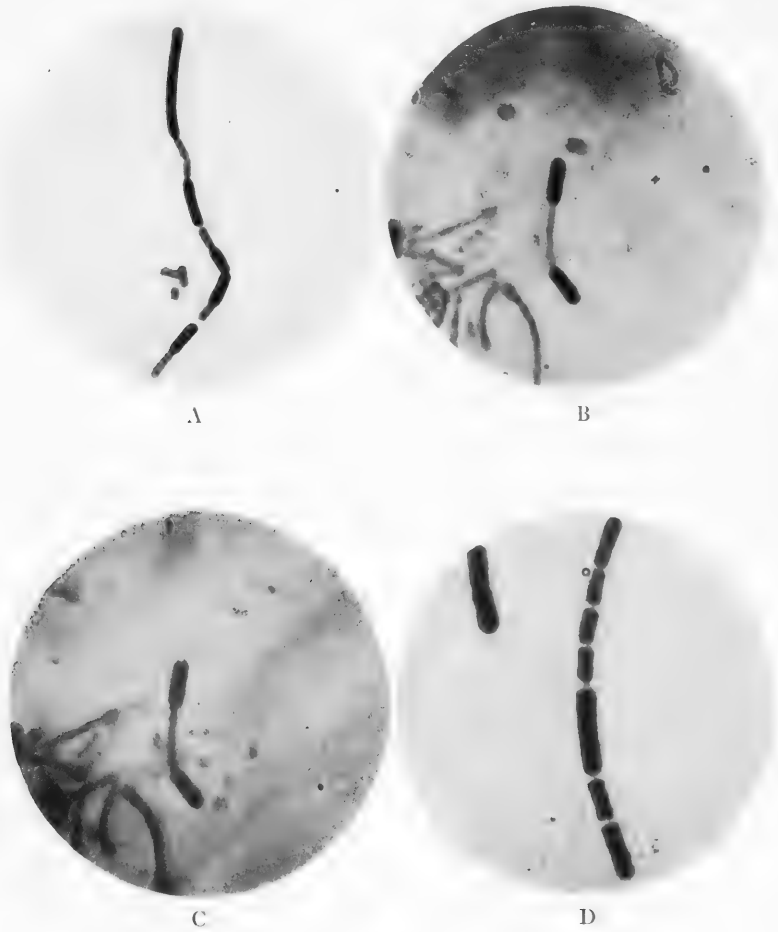

FIG. 5.

(Churchman: *B. anthracis* and Gram reaction.)

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